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UNITED STATES PATENT APPLICATION

OF

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FOR

NOVEL FGF HOMOLOGS

PATENT APPLICATION
96-20C1

Description

NOVEL FGF HOMOLOGS

REFERENCE TO RELATED APPLICATIONS

This application is related to U.S. Patent Application Serial No. 08/951,822, filed on October 16, 1997 and Provisional Application 60/028,646, filed on October 16, 1996, for which claims of benefit are made under 35 U.S.C. § 119(e)(1) and 35 U.S.C. § 120.

BACKGROUND OF THE INVENTION

The fibroblast growth factor (FGF) family consists of at least eighteen distinct members (Basilico et al., Adv. Cancer Res. 59:115-165, 1992 and Fernig et al., Prog. Growth Factor Res. 5(4):353-377, 1994) which generally act as mitogens for a broad spectrum of cell types. For example, basic FGF (also known as FGF-2) is mitogenic *in vitro* for endothelial cells, vascular smooth muscle cells, fibroblasts, and generally for cells of mesoderm or neuroectoderm origin, including cardiac and skeletal myocytes (Gospodarowicz et al., J. Cell. Biol. 70:395-405, 1976; Gospodarowicz et al., J. Cell. Biol. 89:568-578, 1981 and Kardami, J. Mol. Cell. Biochem. 92:124-134, 1990). *In vivo*, bFGF has been shown to play a role in avian cardiac development (Sugi et al., Dev. Biol. 168:567-574, 1995 and Mima et al., Proc. Nat'l. Acad. Sci. 92:467-471, 1995), and to induce coronary collateral development in dogs (Lazarous et al., Circulation 94:1074-1082, 1996). In addition, non-mitogenic activities have been demonstrated for various members of the FGF family. Non-proliferative activities associated with acidic and/or basic FGF include: increased endothelial release of tissue plasminogen activator, stimulation of extracellular matrix synthesis, chemotaxis for endothelial cells, induced

expression of fetal contractile genes in cardiomyocytes (Parker et al., J. Clin. Invest. 85:507-514, 1990), and enhanced pituitary hormonal responsiveness (Baird et al., J. Cellular Physiol. 5:101-106, 1987.)

Several members of the FGF family do not have a signal sequence (aFGF, bFGF and possibly FGF-9) and thus would not be expected to be secreted. In addition, several of the FGF family members have the ability to migrate to the cell nucleus (Friesel et al., FASEB 9:919-925, 1995). All the members of the FGF family bind heparin based on structural similarities. Structural homology crosses species, suggesting a conservation of their structure/function relationship (Ornitz et al., J. Biol. Chem. 271(25):15292-15297, 1996.)

There are four known extracellular FGF receptors (FGFRs), and they are all tyrosine kinases. In general, the FGF family members bind to all of the known FGFRs, however, specific FGFs bind to specific receptors with higher degrees of affinity. Another means for specificity within the FGF family is the spatial and temporal expression of the ligands and their receptors during embryogenesis. Evidence suggests that the FGFs most likely act only in autocrine and/or paracrine manner, due to their heparin binding affinity, which limits their diffusion from the site of release (Flaumenhaft et al., J. Cell. Biol. 111(4):1651-1659, 1990.) Basic FGF lacks a signal sequence, and is therefore restricted to paracrine or autocrine modes of action. It has been postulated that basic FGF is stored intracellularly and released upon tissue damage. Basic FGF has been shown to have two receptor binding regions that are distinct from the heparin binding site (Abraham et al., EMBO J. 5(10):2523-2528, 1986.)

It has been shown that FGFR-3 plays a role in bone growth. Mice made homozygous null for the FGFR-3 (-/-) resulted in postnatal skeletal abnormalities (Colvin

et al., Nature Genet. 12:309-397, 1996 and Deng et al., Cell 84:911-921, 1996). The mutant phenotype suggests that in normal mice, FGFR-3 plays a role in regulation of chrondrocyte cell division in the growth plate region of the bone (Goldfarb, Cytokine and Growth Factor Rev. 7(4):311-325, 1996). The ligand for the FGFR-3 in the bone growth plate has not been identified.

Although four FGFRs have been identified, all of which have been shown to have functional splice variants, the possibility that novel FGF receptors exist is quite likely. For example, no receptor has been identified for the FGF-8a isoform (MacArthur et al., J. Virol. 69(4):2501-2507, 1995.).

FGF-8 is a member of the FGF family that was originally isolated from mammary carcinoma cells as an androgen-inducible mitogen. It has been mapped to human chromosome 10q25-q26 (White et al., Genomics 30:109-11, 1995.) FGF-8 is involved in embryonic limb development (Vogel et al., Development 122:1737-1750, 1996 and Tanaka et al., Current Biology 5(6):594-597, 1995.) Expression of FGF-8 during embryogenesis in cardiac, urogenital and neural tissue indicates that it may play a role in development of these tissues (Crossley et al., Development 121:439-451, 1995.) There is some evidence that acrocephalosyndactylia, a congenital condition marked by peaked head and webbed fingers and toes, is associated with FGF-8 point mutations (White et al., 1995, ibid.)

FGF-8 has five exons, in contrast to the other known FGFs, which have only three exons. The first three exons of FGF-8 correspond to the first exon of the other FGFs (MacArthur et al., Development 121:3603-3613, 1995.) The human gene for FGF-8 codes for four isoforms which differ in their N-terminal regions: FGF isoforms a, b, e, and f; in contrast to the murine gene which gives rise to eight FGF-8 isoforms (Crossley et al., 1995, ibid.) Human FGF-8a and FGF-8b have 100% homology to the murine

proteins, and FGF-8e and FGF-8f proteins are 98% homologous between human and mouse (Gemel et al., Genomics 35:253-257, 1996.)

Heart disease is the major cause of death in the United States, accounting for up to 30% of all deaths. Myocardial infarction (MI) accounts for 750,000 hospital admissions per year in the U.S., with more than 5 million people diagnosed with coronary disease. Risk factors for MI include diabetes mellitus, hypertension, truncal obesity, smoking, high levels of low density lipoprotein in the plasma or genetic predisposition.

Cardiac hyperplasia is an increase in cardiac myocyte proliferation, and has been demonstrated to occur with normal aging in the human and rat (Olivetti et al., J. Am. Coll. Cardiol. 24(1):140-9, 1994 and Anversa et al., Circ. Res. 67:871-885, 1990), and in catecholamine-induced cardiomyopathy in rats (Deisher et al., Am. J. Cardiovasc. Pathol. 5(1):79-88, 1994.) Whether the increase in myocytes originate with some progenitor cell, or are a result of proliferation of a more terminally differentiated cell type, remains controversial.

However, because infarction and other causes of myocardial necrosis appear to be irreparable, it appears that the normal mechanisms of cardiac hyperplasia cannot compensate for extensive myocyte death, and there remains a need for exogenous factors that promote hyperplasia and ultimately result in renewal of the heart's ability to function.

Bone remodeling is the dynamic process by which tissue mass and skeletal architecture are maintained. The process is a balance between bone resorption and bone formation, with two cell types thought to be the major players. These cells are the osteoblast and osteoclast. Osteoblasts synthesize and deposit matrix to become new bone. The activities of osteoblasts and osteoclasts are

regulated by many factors, systemic and local, including growth factors.

While the interaction between local and systemic factors has not been completely elucidated, there does appear to be consensus that growth factors play a key role in the regulation of both normal skeletal remodeling and fracture repair.. Some of the growth factors that have been identified in bone include: IGF-I, IGF-II, TGF-β_1, TGF-β_2, bFGF, aFGF, PDGF and the family of bone morphogenic proteins (Baylink et al., J. Bone Mineral Res. 8 (Supp. 2):S565-S572, 1993).

When bone resorption exceeds bone formation, a net loss in bone results, and the propensity for fractures is increased. Decreased bone formation is associated with aging and certain pathological states. In the U.S. alone, there are approximately 1.5 million fractures annually that are attributed to osteoporosis. The impact of these fractures on the quality of the patient's life is immense. Associated costs to the health care system in the U.S. are estimated to be $5-$10 billion annually, excluding long-term care costs.

Other therapeutic applications for growth factors influencing bone remodeling include, for example, the treatment of injuries which require the proliferation of osteoblasts to heal, such as fractures, as well as stimulation of mesenchymal cell proliferation and the synthesis of intramembraneous bone which have been indicated as aspects of fracture repair (Joyce et al. 36th Annual Meeting, Orthopaedic Research Society, February 5-8, 1990. New Orleans, LA).

The present invention provides such polypeptides for these and other uses that should be apparent to those skilled in the art from the teachings herein.

SUMMARY OF THE INVENTION

The present invention provides an isolated polynucleotide molecule encoding a fibroblast growth factor (FGF) homolog comprising a polynucleotide sequence that encodes for a polypeptide that is at least 80% identical to the amino acid sequence as shown in SEQ ID NO: 2 from amino acid residues 55 to 175.

In other embodiments, the polynucleotide molecule encodes for a polypeptide that is at least 80% identical residues 55 to 196 or 207 of SEQ ID NO: 2.

In another aspect, the present invention provides for an isolated polynucleotide molecule comprising a polynucleotide sequence encoding for a polypeptide that is at least 60% identical to the amino acid sequence as shown in SEQ ID NO: 2 from amino acid residues 28 to 175.

In other embodiments, the polynucleotide molecule encodes for a polypeptide that is at least 80% or 90% identical to the amino acid sequence as shown in SEQ ID NO: 2 from amino acid residues 28 to 175.

In another aspect, the present invention provides for an isolated polynucleotide molecule comprising a polynucleotide sequence encoding for a polypeptide that is at least 60% identical to the amino acid sequence as shown in SEQ ID NO: 2 from amino acid residues 28 to 196.

In other embodiments, the polynucleotide molecule encodes for a polypeptide that is at least 80% or 90% identical to the amino acid sequence as shown in SEQ ID NO: 2 from amino acid residues 28 to 196.

In another aspect, the present invention provides for an isolated polynucleotide molecule comprising a polynucleotide sequence encoding for a polypeptide that is at least 60% identical to the amino acid sequence as shown in SEQ ID NO: 2 from amino acid residues 28 to 207.

In other embodiments, the polynucleotide molecule encodes for a polypeptide that is at least 80% or 90% identical to the amino acid sequence as shown in SEQ ID NO: 2 from amino acid residues 28 to 207.

In other aspects, the present invention provides for a polynucleotide molecule as shown in SEQ ID NO: 1 or SEQ ID NO: 6 from nucleotide 163 or 82 to nucleotide 525 or 585.

In other aspects, the present invention provides expression vectors and cultured cells containing DNA segments comprising the polynucleotide molecules encoding for the FGF homolog polypeptides.

In another aspect, the present invention provides for a method of producing an FGF homolog, wherein the cultured cell expresses polypeptide encoded by polynucleotides comprising the sequences disclosed herein.

In another aspect, the present invention provides an isolated FGF homolog polypeptide comprising an amino acid sequence that is at least 80% identical to the sequence as shown in SEQ ID NO: 2 from residues 55 to 175.

In another aspect, the present invention provides for a polypeptide that is at least 60% identical to the sequence of amino acid residues as shown in SEQ ID NO: 2 from residues 28 to 175.

In other embodiments, the polypeptides are at least 80% or 90% identical to the sequence as shown in SEQ ID NO: 2 from residues 28 to 175.

In another aspect, the present invention provides for a polypeptide that is at least 60% identical to the sequence of amino acid residues as shown in SEQ ID NO: 2 from residues 28 to 196.

In other embodiments, the polypeptides are at least 80% or 90% identical to the sequence as shown in SEQ ID NO: 2 from residues 28 to 196.

In another aspect, the present invention provides for a polypeptide that is at least 60% identical

to the sequence of amino acid residues as shown in SEQ ID NO: 2 from residues 28 to 207.

In other embodiments, the polypeptides are at least 80% or 90% identical to the sequence as shown in SEQ ID NO: 2 from residues 28 to 207.

In another aspect, the present invention provides for pharmaceutical compositions of the FGF homolog polypeptides in combination with a pharmaceutically acceptable vehicle.

In another aspect, the present invention provides for an FGF homolog fusion protein with a first and second portion joined by a peptide bond.

In another aspect, the present invention provides for a method expanding mesenchymal cells population comprising administering an FGF homolog polypeptide as shown in SEQ ID NO: 2 from residues 28 to 175, wherein the polypeptide increases the number of cells as compared to cell populations without the polypeptide.

In other embodiments, the cells are cardiac myocytes, skeletal myocytes, fibroblasts, osteoblasts and pluripotent stem cells.

In another aspect, the present invention provides for a method for improving cardiac performance in a patient by administering a therapeutic amount of an FGF homolog polypeptide as shown in SEQ ID NO: 2 from residue 28 to residue 175 and results in improvement in cardiac performance.

In other embodiments, the cardiac improvement is measure as increase in total ejection fraction, a decrease in end diastolic pressure, an increase in dP/dt or a decrease in vascular resistance.

In other aspect, the present invention provides for increasing cardiac performance in an individual comprising administering an effective amount of a composition comprising an FGF homolog polypeptide as shown

in SEQ ID NO: 2 from residue 28 to residue 175, where the composition results in improved cardiac performance.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 and Figure 2 illustrate a multiple alignment of human fibroblast growth factor homologous factor 1 (FHF-1; SEQ ID NO: 21), human myocyte-activating factor (FGF-10; SEQ ID NO: 22), human fibroblast growth factor homologous factor 4 (FHF-4; SEQ ID NO: 23), human fibroblast growth factor homologous factor 2 (FHF-2; SEQ ID NO: 24), human fibroblast growth factor homologous factor 3 (FHF-3; SEQ ID NO: 25), human FGF-4 (SEQ ID NO: 26), human FGF-6 (SEQ ID NO: 27), human FGF-2 (basic; SEQ ID NO: 28), human FGF-1 (acidic; SEQ ID NO: 29), human keratinocyte growth factor 2 (KGF-2; SEQ ID NO: 30), human keratinocyte growth factor precursor (FGF-7; SEQ ID NO: 31), human zFGF5 (SEQ ID NO: 2), human FGF-8 (SEQ ID NO: 32) human FGF-5 (SEQ ID NO: 33), human FGF-9 (SEQ ID NO: 34), and human FGF-3 (SEQ ID NO: 35). "*" designates conserved amino acids; ":" designates conserved amino acid substitutions; and "." designates less stringently conserved amino acid substitutions.

Figure 3 is an inter-family similarity matrix illustrating the percent identity between: (1) human FGF-5 (SEQ ID NO: 33), (2) human FGF-6 (SEQ ID NO: 27), (3) human FGF-7 (SEQ ID NO: 31), (4) human FGF-8 (SEQ ID NO: 32), (5) human FGF-9 (SEQ ID NO: 34), (6) human zFGF5 (SEQ ID NO: 2), (7) human FGF-10 (SEQ ID NO: 22), (8) human FGF-1 (SEQ ID NO: 29), (9) human FHF-1 (SEQ ID NO: 21), (10) human FGF-2 (SEQ ID NO: 28), (11) human FHF-2 (SEQ ID NO: 24), (12) human FHF-4 (SEQ ID NO: 23), (13) human FGF-3 (SEQ ID NO: 35), (14) human KGF-2 (SEQ ID NO: 30), (15) human FHF-3 (SEQ ID NO: 25), and (16) human FGF-4 (SEQ ID NO: 26).

Figure 4 is a multiple alignment of the amino acid sequences for mature human zFGF5 and mouse zFGF5 (SEQ ID NOS: 2 and 39, respectively).

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention in detail, it may be helpful to the understanding thereof to define the following terms:

The term "affinity tag" is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification or detection of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A (Nilsson et al., EMBO J. 4:1075, 1985; Nilsson et al., Methods Enzymol. 198:3, 1991), glutathione S transferase (Smith and Johnson, Gene 67:31, 1988), Glu-Glu affinity tag (Grussenmeyer et al., Proc. Natl. Acad. Sci. USA 82:7952-4, 1985), substance P, Flag™ peptide (Hopp et al., Biotechnology 6:1204-10, 1988), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general, Ford et al., Protein Expression and Purification 2: 95-107, 1991. DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence. The term allelic variant is also

used herein to denote a protein encoded by an allelic variant of a gene.

The terms "amino-terminal" and "carboxyl-terminal" are used herein to denote positions within polypeptides. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete polypeptide.

The term "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the complement/anti-complement pair preferably has a binding affinity of $<10^{9}$ M^{-1}.

The term "complements of a polynucleotide molecule" is a polynucleotide molecule having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "contig" denotes a polynucleotide that has a contiguous stretch of identical or complementary sequence to another polynucleotide. Contiguous sequences are said to "overlap" a given stretch of polynucleotide sequence either in their entirety or along a partial stretch of the polynucleotide. For example, representative contigs to the polynucleotide sequence 5'-

ATGGCTTAGCTT-3' are 5'-TAGCTTgagtct-3' and 3'-gtcgacTACCGA-5'.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

The term "expression vector" is used to denote a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments include promoter and terminator sequences, and may also include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, etc. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

The term "isolated", when applied to a polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, <u>Nature 316</u>:774-78, 1985).

An "isolated" polypeptide or protein is a polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and

animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

The term "operably linked", when referring to DNA segments, indicates that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates in the promoter and proceeds through the coding segment to the terminator.

The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

A "polynucleotide" is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be

understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

The term "promoter" is used herein for its art-recognized meaning to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule (i.e., a ligand) and mediates the effect of the ligand on the cell. Membrane-bound receptors are characterized by a multi-peptide structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. Binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the

effector domain and other molecule(s) in the cell. This interaction in turn leads to an alteration in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. In general, receptors can be membrane bound, cytosolic or nuclear; monomeric (e.g., thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (e.g., PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor).

The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

The term "splice variant" is used herein to denote alternative forms of RNA transcribed from a gene. Splice variation arises naturally through use of alternative splicing sites within a transcribed RNA molecule, or less commonly between separately transcribed RNA molecules, and may result in several mRNAs transcribed from the same gene. Splice variants may encode polypeptides having altered amino acid sequence. The term splice variant is also used herein to denote a protein encoded by a splice variant of an mRNA transcribed from a gene.

Molecular weights and lengths of polymers determined by imprecise analytical methods (e.g., gel electrophoresis) will be understood to be approximate values. When such a value is expressed as "about" X or

"approximately" X, the stated value of X will be understood to be accurate to ±10%.

All references cited herein are incorporated by reference in their entirety.

The present invention is based in part upon the discovery of a novel DNA sequence that encodes a fibroblast growth factor (FGF) homolog polypeptide having homology to FGF-8 and FGF-17 (Hoshikawa et al., Biochem. Biophys. Res. Comm. 244:187-191, 1998). Analysis of the tissue distribution of the human mRNA corresponding to this novel DNA showed that expression was highest in fetal heart tissue and adult heart tissue, followed by apparent but decreased expression levels in fetal lung, skeletal muscle, smooth muscle tissues such as small intestine, colon and trachea. The FGF homolog polypeptide has been designated zFGF5.

Tissue distribution in murine species does not appear to completely correspond with expression in human tissues. Northern analysis of mouse tissues revealed that expression of mouse zFGF5 is highest in spleen and day 17 embryo, followed by relatively lower expression in heart, lung, kidney and testis. Mouse heart tissue analysis found expression highest in day 16 fetal heart tissue, with expression in adult heart present in most mouse strains. It also appears that there may be variability within murine expression levels and tissues (Hu et al., Mol. Cell. Biol. 18:6063-6074, 1998; Ohbayashi et al., J. Biol. Chem. 273:18161-18164, 1998 and Maruoka et al., Mech. Develop. 74:175-175, 1998).

The novel human zFGF5 polypeptides of the present invention were initially identified by querying an EST database for growth factors. A single EST sequence was discovered and predicted to be related to the FGF family. The novel FGF homolog polypeptide encoded by the full length cDNA contained a motif of the formula:

CXFXEX{6}Y, wherein X is any amino acid and X{} is the number of X amino acids greater than one (SEQ ID NO: 36). This motif occurs in all known members of the FGF family and is unique to these proteins.

The nucleotide sequence of the zFGF5 cDNA is described in SEQ ID NO. 1, and its deduced amino acid sequence is described in SEQ ID NO. 2. When amino acid residue 28 (Glu) to amino acid residue 181 (Gln) of SEQ ID NO: 2 is compared to the corresponding region of FGF-8 (See Figures 1 and 2) the aligned and deduced amino acid sequence has approximately 56% identity. FGF-17 (Hoshiwara et al., Biochem. Biophys. Res. Comm. 244:187-191, 1998) has recently been identified, and has the highest degree of homology to zFGF5. The region of highest identity is ~66% over a 123 amino acid overlap which corresponds to the region of SEQ ID NO: 2 from residue 55 (Tyr) to residue 177 (Arg).

The novel polypeptide encoded by the polynucleotide described herein contains the CXFXE{6}Y motif present in all members of the FGF family. The CXFXE{6}Y motifs (SEQ ID NO: 36) are highly conserved. A consensus amino acid sequence of the CXFXEX{6}Y domain (SEQ ID NO: 36) includes human fibroblast growth factor homologous factor 1 (FHF-1; Smallwood et al., Proc. Natl. Acad. Sci. USA 93:9850-9857, 1996), human myocyte-activating factor (FGF-10; HSU76381, GENBANK identifier, http://www.ncbi.nlm.nih.gov/), human fibroblast growth factor homologous factor 4 (FHF-4; Smallwood et al., 1996, *ibid.*), human fibroblast growth factor homologous factor 2 (FHF-2; Smallwood et al., 1996, *ibid.*), human fibroblast growth factor homologous factor 3 (FHF-3; Smallwood et al., 1996, *ibid.*), human FGF-4 (Basilico et al., Adv. Cancer Res. 59:115-165,1992), human FGF-6 (Basilico et al., 1992, *ibid.*), human FGF-2 (basic; Basilico et al., 1992, *ibid.*), human FGF-1 (acidic; Basilico et al., 1992, *ibid.*), human keratinocyte growth factor 2 (KGF-2;

HSU67918 GENBANK identifier, http://www.ncbi.nlm.nih.gov/), human keratinocyte growth factor precursor (FGF-7; Basilico et al., 1992, *ibid.*), human zFGF5, human FGF-8 (Gemel et al., Genomics 35:253-257, 1996), human FGF-5 (Basilico et al., 1992, *ibid.*), human FGF-9 (Miyamoto et al., Mol. Cell. Biol. 13:4251-4259, 1993), human FGF-3 (Basilico et al., 1992, *ibid.*), and FGF-17 (Hoshiwara et al., 1998, *ibid.*).

Analysis of the cDNA encoding a zFGF5 polypeptide (SEQ ID NO: 1) revealed an open reading frame encoding 207 amino acids (SEQ ID NO: 2) comprising a mature polypeptide of 180 amino acids (residue 28 to residue 207 of SEQ ID NO: 2). Multiple alignment of zFGF5 with other known FGFs revealed a block of high percent identity corresponding to amino acid residue 127 (Cys) to amino acid residue 138 (Tyr), of SEQ ID NO: 2 and is shown in Figure 1. Several of the members of the FGF family do not have signal sequences.

The mouse zFGF5 cDNA was cloned using PCR with a mouse embryo cDNA library as a template and oligonucleotide primers designed from the 5' and 3' ends of the human zFGF5 cDNA. The mouse zFGF5 polynucleotide sequence as shown in SEQ ID NO: 38 and corresponding amino acid sequence as shown in SEQ ID NO: 39 were found to have a high degree of homology. At the amino acid level, the mouse and human polypeptides are approximately 98% identical, with three amino acid changes. The changes as shown in Figure 4, correspond to a Val_{26} in SEQ ID NO: 2 being Ala_{26} in SEQ ID NO: 39 in the mouse polypeptide, Pro_{183} in SEQ ID NO: 2 to Ala_{183} in SEQ ID NO: 39 and Ala_{207} in SEQ ID NO: 2 to Gly_{207} in SEQ ID NO: 39. As is noted previously, Ala_{26} (mouse) and the corresponding Val_{26} (human) are in the secretory signal sequence, leaving only two amino acid differences in the mature polypeptide. Based on the high identity between the mouse and human sequences, it is predicted that function will be

equivalent as well. However, based on differences in tissue distribution for the mouse and human expression, zFGF5 may have a wider organ target distribution, and more diverse biological functions in the mouse than in the human.

Members of the FGF family are characterized by heparin binding domains. A putative heparin-binding domain for zFGF5 has been identified in the region of amino acid residue 148 (Gly) to amino acid residue 169 (Gln) of SEQ ID NO: 2 and SEQ ID NO: 39.

It is postulated that receptor-mediated signaling is initiated upon binding of FGF ligand complexed with cell-surface heparin sulfate proteoglycans. Many FGF family members can be placed into one of two related families on the basis of their structures and functions. aFGF and bFGF consist of three exons separated by two introns of variable length. FGF-8 consists of five exons, the first three of which correspond to the first exon of aFGF and bFGF. All the known FGF family members are spliced to form single polypeptides.

SEQ ID NO: 6 is a degenerate polynucleotide sequence that encompasses all polynucleotides that could encode the zFGF5 polypeptide of SEQ ID NO: 2 (amino acids 1 or 28 to 207). Thus, zFGF5 polypeptide-encoding polynucleotides ranging from nucleotide 1 or 82 to nucleotide 621 of SEQ ID NO: 6 are contemplated by the present invention. Also contemplated by the present invention are fragments and fusions as described above with respect to SEQ ID NO: 1, which are formed from analogous regions of SEQ ID NO: 6, wherein nucleotides 82 to 621 of SEQ ID NO: 6 correspond to nucleotides 82 to 621 of SEQ ID NO: 1, for the encoding a mature zFGF5 molecule.

The symbols in SEQ ID NO: 6 are summarized in Table 1 below.

TABLE 1

<table>
<tr><th>Nucleotide</th><th>Resolutions</th><th>Complement</th><th>Resolutions</th></tr>
<tr><td>A</td><td>A</td><td>T</td><td>T</td></tr>
<tr><td>C</td><td>C</td><td>G</td><td>G</td></tr>
<tr><td>G</td><td>G</td><td>C</td><td>C</td></tr>
<tr><td>T</td><td>T</td><td>A</td><td>A</td></tr>
<tr><td>R</td><td>A|G</td><td>Y</td><td>C|T</td></tr>
<tr><td>Y</td><td>C|T</td><td>R</td><td>A|G</td></tr>
<tr><td>M</td><td>A|C</td><td>K</td><td>G|T</td></tr>
<tr><td>K</td><td>G|T</td><td>M</td><td>A|C</td></tr>
<tr><td>S</td><td>C|G</td><td>S</td><td>C|G</td></tr>
<tr><td>C|G</td><td>A|T</td><td>W</td><td>A|T</td></tr>
<tr><td>H</td><td>A|C|T</td><td>D</td><td>A|G|T</td></tr>
<tr><td>B</td><td>C|G|T</td><td>V</td><td>A|C|G</td></tr>
<tr><td>V</td><td>A|C|G</td><td>B</td><td>C|G|T</td></tr>
<tr><td>D</td><td>A|G|T</td><td>H</td><td>A|C|T</td></tr>
<tr><td>N</td><td>A|C|G|T</td><td>N</td><td>A|C|G|T</td></tr>
</table>

The degenerate codons used in SEQ ID NO: 6, encompassing all possible codons for a given amino acid, are set forth in Table 2 below.

TABLE 2

Amino Acid	Letter	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn|Asp	B		RAY
Glu|Gln	Z		SAR
Any	X		NNN
Gap	-	---	

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding each amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode

arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may have some incorrect amino acids, but one of ordinary skill in the art can easily identify such erroneous sequences by reference to the amino acid sequence of SEQ ID NO: 2.

The highly conserved amino acids in zFGF5 can be used as a tool to identify new family members. To identify new family members in EST databases, the conserved CXFXEX{6}Y motif (SEQ ID NO: 36) can be used. In another method using polynucleotide probes and hybridization methods, RNA obtained from a variety of tissue sources can be used to generate cDNA libraries and probe these libraries for new family members. In particular, reverse transcription-polymerase chain reaction (RT-PCR) can be used to amplify sequences encoding highly degenerate DNA primers designed from the sequences corresponding to amino acid residue 127 (Cys) to amino acid residue 138 (Tyr) of SEQ ID NO: 2.

Within preferred embodiments of the invention the isolated polynucleotides will serve as a probe and hybridize to similar sized regions of SEQ ID NO: 1 or a sequence complementary thereto, under stringent conditions. In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typical stringent conditions are those in which the salt concentration is at least about 0.02 M at pH 7 and the temperature is at least about 60°C.

As previously noted, the isolated polynucleotides of the present invention include DNA and

RNA. Methods for isolating DNA and RNA are well known in the art. It is generally preferred to isolate RNA from cardiac tissue, although DNA can also be prepared using RNA from other tissues or isolated as genomic DNA. Total RNA can be prepared using guanidine HCl extraction followed by isolation by centrifugation in a CsCl gradient (Chirgwin et al., Biochemistry 18:52-94, 1979). Poly $(A)^+$ RNA is prepared from total RNA using the method of Aviv and Leder (Proc. Natl. Acad. Sci. USA 69:1408-1412, 1972). Complementary DNA (cDNA) is prepared from poly$(A)^+$ RNA using known methods. Polynucleotides encoding zFGF5 polypeptides are then identified and isolated by, for example, hybridization or PCR.

The present invention further provides counterpart polypeptides and polynucleotides from other species (orthologs). Of particular interest are zFGF5 polypeptides from other mammalian species, including murine, rat, porcine, ovine, bovine, canine, feline, equine and other primate proteins. Identification of variants of the human sequence are particularly interesting because while 8 variants of murine FGF-8 have been identifed, only 4 human variants are known. Human variants or orthologs of the human proteins can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses the protein. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A zFGF5-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also be cloned using the polymerase chain reaction, or PCR

(Mullis, U.S. Patent 4,683,202), using primers designed from the sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to zFGF5. Similar techniques can also be applied to the isolation of genomic clones.

Those skilled in the art will recognize that the sequences disclosed in SEQ ID NO: 1 or SEQ ID NO: 38 and SEQ ID NO: 2 and SEQ ID NO: 39 represent a single allele of the human and mouse zFGF5 gene and polypeptide, respectively, and that allelic variation and alternative splicing are expected to occur. Allelic variants can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of the DNA sequence shown in SEQ ID NO: 1 or SEQ ID NO: 38, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NO: 2 or SEQ ID NO: 39.

The present invention also provides isolated zFGF5 polypeptides that are substantially homologous to the polypeptides of SEQ ID NO: 2 and their orthologs. The term "substantially homologous" is used herein to denote polypeptides having 50%, preferably 60%, more preferably at least 80%, sequence identity to the sequences shown in SEQ ID NO: 2 or their orthologs. Such polypeptides will more preferably be at least 90% identical, and most preferably 95% or more identical to SEQ ID NO: 2 or its orthologs. Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., Bull. Math. Bio. 48: 603-616, 1986 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-10919, 1992. Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a

gap extension penalty of 1, and the "blosum 62" scoring matrix of Henikoff and Henikoff (ibid.) as shown in Table 3 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as:

Table 3

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

$$\frac{\text{Total number of identical matches}}{\text{[length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences]}} \times 100$$

Sequence identity of polynucleotide molecules is determined by similar methods using a ratio as disclosed above.

Substantially homologous proteins and polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 4) and other substitutions that do not significantly affect the folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification (an affinity tag), such as a poly-histidine tract, protein A (Nilsson et al., EMBO J. 4:1075, 1985; Nilsson et al., Methods Enzymol. 198:3, 1991), glutathione S transferase (Smith and Johnson, Gene 67:31, 1988), maltose binding protein (Kellerman and Ferenci, Methods Enzymol. 90:459-463, 1982; Guan et al., Gene 67:21-30, 1987), or other antigenic epitope or binding domain. See, in general Ford et al., Protein Expression and Purification 2: 95-107, 1991, which is incorporated herein by reference. DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ; New England Biolabs, Beverly, MA).

Table 4

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

The proteins of the present invention can also comprise, in addition to the 20 standard amino acids, non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, trans-3-methylproline, 2,4-methanoproline, cis-4-hydroxyproline, trans-4-hydroxyproline, N-methyl-glycine, allo-threonine, methylthreonine, hydroxyethyl-cysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipecolic acid, tert-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenyl-alanine, 4-fluorophenylalanine, 4-hydroxyproline, 6-*N*-methyl lysine, 2-aminoisobutyric acid, isovaline and α-methyl serine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be

employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations are carried out in a cell free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., J. Am. Chem. Soc. 113:2722, 1991; Ellman et al., Meth. Enzymol. 202:301, 1991; Chung et al., Science 259:806-09, 1993; and Chung et al., Proc. Natl. Acad. Sci. USA 90:10145-49, 1993). In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., J. Biol. Chem. 271:19991-98, 1996). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide et al., Biochem. 33:7470-76, 1994. Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, Protein Sci. 2:395-403, 1993).

Essential amino acids in the zFGF5 polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244: 1081-1085, 1989). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant

molecules are tested for biological activity (e.g., receptor binding activity using ^{125}I-zFGF5 (Moscatelli, J. Cell Physio. 131:123-130. 1987), activation of receptor tyrosine kinase (Panek et al., J. Pharm. Exp. Therapeutics 286:569-577, 1998 and Schafer et al., Anal. Biochem. 261:100-112, 1998), generation of cardiac myocytes or fibroblasts, or stimulation of bone formation) to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton et al., J. Biol. Chem. 271:4699-4708, 1996. Sites of ligand-receptor interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., Science 255:306-312, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992. The identities of essential amino acids can also be inferred from analysis of homologies with related FGFs and are shown in Figures 1 and 2.

Analyses of the amino acid sequence of human and mouse zFGF5 revealed a dibasic site at the C-terminus of the polypeptide (amino acid residue 196-197 (Lys-Arg)). A C-terminally truncated polypeptide comprising an amino acid sequence as shown in SEQ ID NO: 2, from amino acid residue 28 (Glu) to amino acid residue 196 (Lys) was demonstrated to have biological activity. Dibasic amino acids, such as, Arg-X-X-Arg (wherein X is any amino acid residue; SEQ ID NO: 37), Arg-Arg or Lys-Arg; are subject to cleavage by several enzymes, including, but not limited to, thrombin and carboxypeptidases. Therefore, it is within the scope of the claims to make conservative changes at dibasic amino acid residues, in particular the dibasic residues at amino acid residues 196 and 197 (Lys and Arg, respectively) of SEQ ID NO: 2 or SEQ ID NO: 39.

Based on analyses of the FGF family a C-terminally truncated molecule that comprises amino acid residue 28 (Glu) to residue 175 (Met) of SEQ ID NO: 2 may be biologically active. An intramolecular disulfide bond is predicted to occur between amino acid residue 109 (Cys) and residue 127 (Cys) of SEQ ID NO: 2 or SEQ ID NO: 39.

Based on homology alignments with FGF-1 and FGF-2 crystal structures (Eriksson et al., Prot. Sci. 2:1274, 1993), secondary structure predictions for beta strand structure of zFGF5 correlates to amino acid residues 56-59, 64-69, 73-76, 85-92, 96-102, 106-111, 115-119, 128-134, 138-144, 149-155, and 173-177 of SEQ ID NO: 2 or SEQ ID NO: 39. Amino acids critical for zFGF5 binding to receptors can be identified by site-directed mutagenesis of the entire zFGF5 polypeptide. More specifically, they can be identified using site-directed mutagenesis of amino acids in the zFGF5 polypeptide which correspond to amino acid residues in acidic FGF (FGF1) and basic FGF (FGF2) identified as critical for binding of these FGFs to their receptors (Blaber et al., Biochem. 35:2086-2094, 1996). These amino acids include Tyr33, Arg53, Asn110, Tyr112, Lys119, Trp123, Leu149 and Met151 in human FGF2, and Tyr30, Arg50, Asn107, Tyr109, Lys116, Trp122, Leu148 and Leu150 in human FGF1, as shown in Fig.1 and Fig.2. The corresponding amino acids in zFGF5, as shown in Fig.1 and Fig.2, would be Tyr58, Gly77, Asn136, Tyr138, Lys145, Trp149, Met175 and Arg177. One skilled in the art will recognize that other members, in whole or in part, of the FGF family may have structural or biochemical similarities to zFGF5, and be substituted making such analyses. Such regions would be important for biological functions of the molecule.

An alignment based on homology of zFGF5 with FGF-17 revealed the highest percent identity region consists of a 123 amino acid overlap found between residue 55 (Tyr) and residue 177 (Arg) of SEQ ID NO: 2 with ~66%

identity. When conservative amino acid changes are calculated over the same region, the percent homology is ~ 92%.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53-57, 1988) or Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-2156, 1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-10837, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).

Mutagenesis methods as disclosed above can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode active polypeptides (e.g., cell proliferation) can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

Using the methods discussed above, one of ordinary skill in the art can identify and/or prepare a variety of polypeptides that are substantially homologous to residues 28 (Glu) to 196 (Lys) or residues 28 (Glu) to 207 (Ala) of SEQ ID NO: 2, allelic variants thereof, or biologically active fragments thereof, and retain the proliferative properties of the wild-type protein. Such

polypeptides may also include additional polypeptide segments as generally disclosed above.

The polypeptides of the present invention, including full-length proteins, fragments thereof and fusion proteins, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987, which are incorporated herein by reference.

In general, a DNA sequence encoding a zFGF5 polypeptide of the present invention is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a zFGF5 polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be the native sequence, or a chimera comprising a signal sequence derived from another secreted protein (e.g., t-PA and α-pre-pro secretory leader) or synthesized *de novo*. The secretory signal sequence is joined to the zFGF5 DNA sequence in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Fungal cells, including yeast cells, can also be used within the present invention. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in *Saccharomyces cerevisiae* is the *POT1* vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman et al., U.S. Patent No.

4,615,974; and Bitter, U.S. Patent No. 4,977,092), and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., J. Gen. Microbiol. 132:3459-3465, 1986 and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

The use of *Pichia methanolica* as host for the production of recombinant proteins is disclosed in WIPO Publications WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565. DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For polypeptide production in *P. methanolica*, it is preferred that the promoter and terminator in the plasmid be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (*AUG1* or *AUG2*). Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the plasmid flanked at both ends by host DNA sequences. A preferred selectable marker for use in *Pichia methanolica* is a *P. methanolica ADE2* gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21), which allows *ade2* host cells to grow in the

absence of adenine. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is preferred to use host cells in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are preferred. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. It is preferred to transform *P. methanolica* cells by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (τ) of from 1 to 40 milliseconds, most preferably about 20 milliseconds.

Other methods for transforming yeast cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075, which are incorporated herein by reference. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). An alternative preferred vector system for use in yeast is the POT1 vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092, which are incorporated herein by reference) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454, which are incorporated herein by reference. Transformation

systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia guillermondii*, and *Candida maltosa* are known in the art. A particularly preferred system utilizes *Pichia methanolica* (see, PCT application WO 9717450). For alternative transformation systems, see, for example, Gleeson et al., J. Gen. Microbiol. 132:3459-3465, 1986 and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349, which is incorporated herein by reference. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228, which is incorporated herein by reference. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533, which is incorporated herein by reference.

Cultured mammalian cells are also preferred hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981: Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987), and liposome-mediated transfection (Hawley-Nelson et al., Focus 15:73, 1993; Ciccarone et al., Focus 15:80, 1993), which are incorporated herein by reference. The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134, which are incorporated herein by reference. Preferred cultured

mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) and Chinese hamster ovary (e.g. CHO-K1; ATCC No. CCL 61 or DG44) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978, which are incorporated herein by reference) and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems may also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g., hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used.

Other higher eukaryotic cells can also be used as hosts, including insect cells, plant cells and avian cells. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino et al., U.S. Patent No. 5,162,222; Bang et al., U.S. Patent No. 4,775,624; and WIPO publication WO 94/06463, which are incorporated herein by reference. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., J. Biosci. (Bangalore) 11:47-58, 1987.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell.

Expressed recombinant zFGF5 polypeptides (or chimeric zFGF5 polypeptides) can be purified using fractionation and/or conventional purification methods and media. Ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable anion exchange media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred, with DEAE Fast-Flow Sepharose (Pharmacia, Piscataway, NJ)

being particularly preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties. Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers. Methods for binding receptor polypeptides to support media are well known in the art. Selection of a particular method is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988.

The polypeptides of the present invention can also be isolated by exploitation of their heparin binding properties. For a review, see, Burgess et al., Ann. Rev. of Biochem. 58:575-606, 1989. Members of the FGF family can be purified to apparent homogeneity by heparin-Sepharose affinity chromatography (Gospodarowicz et al., Proc. Natl. Acad. Sci. 81:6963-6967, 1984) and eluted using linear step gradients of NaCl (Ron et al., J. Biol. Chem. 268(4):2984-2988, 1993; Chromatography: Principles

& Methods, pp. 77-80, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1993; in "Immobilized Affinity Ligand Techniques", Hermanson et al., eds., pp. 165-167, Academic Press, San Diego, 1992; Kjellen et al., Ann. Rev. Biochem.Ann. Rev. Biochem. 60:443-474, 1991; and Ke et al., Protein Expr. Purif. 3(6):497-507, 1992.)

Other purification methods include using immobilized metal ion adsorption (IMAC) chromatography to purify histidine-rich proteins. Briefly, a gel is first charged with divalent metal ions to form a chelate (E. Sulkowski, Trends in Biochem. 3:1-7, 1985). Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography (Methods in Enzymol., Vol. 182, "Guide to Protein Purification", M. Deutscher, (ed.), Acad. Press, San Diego, 1990, pp.529-39). Alternatively, a fusion of the polypeptide of interest and an affinity tag (e.g., polyhistidine, maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification.

Protein refolding (and optionally reoxidation) procedures may be advantageously used. It is preferred to purify the protein to >80% purity, more preferably to >90% purity, even more preferably >95%, and particularly preferred is a pharmaceutically pure state, that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. Preferably, a purified protein is substantially free of other proteins, particularly other proteins of animal origin.

zFGF5 polypeptides or fragments thereof may also be prepared through chemical synthesis. zFGF5 polypeptides

may be monomers or multimers; glycosylated or non-glycosylated; pegylated or non-pegylated; and may or may not include an initial methionine amino acid residue.

The activity of molecules of the present invention can be measured using a variety of assays that, for example, measure neogenesis or hyperplasia (i.e., proliferation) of cardiac cells based on the tissue specificity in adult heart. Additional activities likely associated with the polypeptides of the present invention include proliferation of endothelial cells, cardiomyocytes, fibroblasts, skeletal myocytes directly or indirectly through other growth factors; action as a chemotaxic factor for endothelial cells, fibroblasts and/or phagocytic cells; osteogenic factor; and factor for expanding mesenchymal stem cell and precursor populations.

Proliferation can be measured using cultured cardiac cells or *in vivo* by administering molecules of the claimed invention to the appropriate animal model. Generally, proliferative effects are seen as an increase in cell number and therefore, may include inhibition of apoptosis, as well as mitogenesis. Cultured cells include cardiac fibroblasts, cardiac myocytes, skeletal myocytes, human umbilical endothelial vein cells from primary cultures. Established cell lines include: NIH 3T3 fibroblast (ATCC No. CRL-1658), CHH-1 chum heart cells (ATCC No. CRL-1680), H9c2 rat heart myoblasts (ATCC No. CRL-1446), Shionogi mammary carcinoma cells (Tanaka et al., Proc. Natl. Acad. Sci. 89:8928-8932, 1992) and LNCap.FGC adenocarcinoma cells (ATCC No. CRL-1740.) Assays measuring cell proliferation are well known in the art. For example, assays measuring proliferation include such assays as chemosensitivity to neutral red dye (Cavanaugh et al., Investigational New Drugs 8:347-354, 1990, incorporated herein by reference), incorporation of radiolabelled nucleotides (Cook et al., Analytical Biochem. 179:1-7, 1989, incorporated herein by reference),

incorporation of 5-bromo-2'-deoxyuridine (BrdU) in the DNA of proliferating cells (Porstmann et al., J. Immunol. Methods 82:169-179, 1985, incorporated herein by reference), and use of tetrazolium salts (Mosmann, J. Immunol. Methods 65:55-63, 1983; Alley et al., Cancer Res. 48:589-601, 1988; Marshall et al., Growth Reg. 5:69-84, 1995; and Scudiero et al., Cancer Res. 48:4827-4833, 1988; all incorporated herein by reference).

Differentiation is a progressive and dynamic process, beginning with pluripotent stem cells and ending with terminally differentiated cells. Pluripotent stem cells that can regenerate without commitment to a lineage express a set of differentiation markers that are lost when commitment to a cell lineage is made. Progenitor cells express a set of differentiation markers that may or may not continue to be expressed as the cells progress down the cell lineage pathway toward maturation. Differentiation markers that are expressed exclusively by mature cells are usually functional properties such as cell products, enzymes to produce cell products and receptors. The stage of a cell population's differentiation is monitored by identification of markers present in the cell population. Myocytes, osteoblasts, adipocytes, chrondrocytes, fibroblasts and reticular cells are believed to originate from a common mesenchymal stem cell (Owen et al., Ciba Fdn. Symp. 136:42-46, 1988). Markers for mesenchymal stem cells have not been well identified (Owen et al., J. of Cell Sci. 87:731-738, 1987), so identification is usually made at the progenitor and mature cell stages. The existence of early stage cardiac myocyte progenitor cells (often referred to as cardiac myocyte stem cells) has been speculated, but not demonstrated, in adult cardiac tissue. However, recent evidence confirms the presence of myocyte proliferation in end-stage cardiac failure in humans (Kajstura et al., Proc. Natl. Assoc. Science, 95:8801-8805, 1998). The

novel polypeptides of the present invention are useful to isolate mesenchymal stem cells and cardiac myocyte progenitor cells, both *in vivo* and *ex vivo*.

There is evidence to suggest that factors that stimulate specific cell types down a pathway towards terminal differentiation or dedifferentiation, affects the entire cell population originating from a common precursor or stem cell. Thus, the present invention includes stimulating inhibition or proliferation of myocytes, smooth muscle cells, osteoblasts, adipocytes, chrondrocytes and endothelial cells. Molecules of the present invention may, while stimulating proliferation or differentiation of cardiac myocytes, inhibit proliferation or differentiation of adipocytes, by virtue of the affect on their common precursor/stem cells. Thus molecules of the present invention, have use in inhibiting chondrosarcomas, atherosclerosis, restenosis, osteoporosis and obesity.

Assays measuring differentiation include, for example, measuring cell-surface markers associated with stage-specific expression of a tissue, enzymatic activity, functional activity or morphological changes (Watt, FASEB, 5:281-284, 1991; Francis, Differentiation 57:63-75, 1994; Raes, Adv. Anim. Cell Biol. Technol. Bioprocesses, 161-171, 1989; all incorporated herein by reference).

In vivo assays for evaluating cardiac neogenesis or hyperplasia include treating neonatal and mature rats with the molecules of the present invention. The animals cardiac function is measured as heart rate, blood pressure, and cardiac output to determine left ventricular function. Post-mortem methods for assessing cardiac improvement include: increased cardiac weight, nuclei/cytoplasmic volume, staining of cardiac histology sections to determine proliferating cell nuclear antigen (PCNA) vs. cytoplasmic actin levels (Quaini et al.,

Circulation Res. 75:1050-1063, 1994 and Reiss et al., Proc. Natl. Acad. Sci. 93:8630-8635, 1996.)

In vivo assays for measuring changes in bone formation rates include performing bone histology (see, Recker, R., eds. Bone Histomorphometry: Techniques and Interpretation. Boca Raton: CRC Press, Inc., 1983) and quantitative computed tomography (QCT; Ferretti,J. Bone 17:353S-364S, 1995; Orphanoludakis et al., Investig. Radiol. 14:122-130,, 1979 and Durand et al., Medical Physics 19:569-573, 1992). An *ex vivo* assay for measuring changes in bone formation would be, for example, a calavarial assay (Gowen et al., J. Immunol. 136:2478-2482, 1986).

With regard to modulating energy balance, particularly as it relates to adipocyte metabolism, proliferation and differentiation, zFGF5 polypeptides modulate effects on metabolic reactions. Such metabolic reactions include adipogenesis, gluconeogenesis, glycogenolysis, lipogenesis, glucose uptake, protein synthesis, thermogenesis, oxygen utilization and the like. Among other methods known in the art or described herein, mammalian energy balance may be evaluated by monitoring one or more of the aforementioned metabolic functions. These metabolic functions are monitored by techniques (assays or animal models) known to one of ordinary skill in the art, as is more fully set forth below. For example, the glucoregulatory effects of insulin are predominantly exerted in the liver, skeletal muscle and adipose tissue. In skeletal muscle and adipose tissue, insulin acts to stimulate the uptake, storage and utilization of glucose.

Art-recognized methods exist for monitoring all of the metabolic functions recited above. Thus, one of ordinary skill in the art is able to evaluate zFGF5 polypeptides, fragments, fusion proteins, antibodies, agonists and antagonists for metabolic modulating

functions. Exemplary modulating techniques are set forth below.

Insulin-stimulated lipogenesis, for example, may be monitored by measuring the incorporation of ^{14}C-acetate into triglyceride (Mackall et al. J. Biol. Chem. 251:6462-6464, 1976) or triglyceride accumulation (Kletzien et al., Mol. Pharmacol. 41:393-398, 1992).

zFGF5-stimulated uptake may be evaluated, for example, in an assay for insulin-stimulated glucose transport. Primary adipocytes or NIH 3T3 L1 cells (ATCC No. CCL-92.1) are placed in DMEM containing 1 g/l glucose, 0.5 or 1.0% BSA, 20 mM Hepes, and 2 mM glutamine. After two to five hours of culture, the medium is replaced with fresh, glucose-free DMEM containing 0.5 or 1.0% BSA, 20 mM Hepes, 1 mM pyruvate, and 2 mM glutamine. Appropriate concentrations of zFGF5, insulin or IGF-1, or a dilution series of the test substance, are added, and the cells are incubated for 20-30 minutes. ^{3}H or ^{14}C-labeled deoxyglucose is added to ≈50 μM final concentration, and the cells are incubated for approximately 10-30 minutes. The cells are then quickly rinsed with cold buffer (e.g. PBS), then lysed with a suitable lysing agent (e.g. 1% SDS or 1 N NaOH). The cell lysate is then evaluated by counting in a scintillation counter. Cell-associated radioactivity is taken as a measure of glucose transport after subtracting non-specific binding as determined by incubating cells in the presence of cytochalasin b, an inhibitor of glucose transport. Other methods include those described by, for example, Manchester et al., Am. J. Physiol. 266 (Endocrinol. Metab. 29):E326-E333, 1994 (insulin-stimulated glucose transport).

Protein synthesis may be evaluated, for example, by comparing precipitation of ^{35}S-methionine-labeled proteins following incubation of the test cells with ^{35}S-methionine and ^{35}S-methionine and a putative modulator of protein synthesis.

Thermogenesis may be evaluated as described by B. Stanley in *The Biology of Neuropeptide Y and Related Peptides*, W. Colmers and C. Wahlestedt (eds.), Humana Press, Ottawa, 1993, pp. 457-509; C. Billington et al., Am. J. Physiol. 260:R321, 1991; N. Zarjevski et al., Endocrinology 133:1753, 1993; C. Billington et al., Am. J. Physiol. 266:R1765, 1994; Heller et al., Am. J. Physiol. 252(4 Pt 2): R661-7, 1987; and Heller et al., Am. J. Physiol. 245(3): R321-8, 1983. Also, metabolic rate, which may be measured by a variety of techniques, is an indirect measurement of thermogenesis.

Oxygen utilization may be evaluated as described by Heller et al., Pflugers Arch. 369(1): 55-9, 1977. This method also involved an analysis of hypothalmic temperature and metabolic heat production. Oxygen utilization and thermoregulation have also been evaluated in humans as described by Haskell et al., J. Appl. Physiol. 51(4): 948-54, 1981.

zFGF5 polypeptides can also be used to prepare antibodies that specifically bind to zFGF5 epitopes, peptides or polypeptides. Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; and Hurrell, J. G. R., Ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, FL, 1982, which are incorporated herein by reference). As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from a variety of warm-blooded animals, such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats.

The immunogenicity of a zFGF5 polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of zFGF5 or a

portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as $F(ab')_2$ and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included. Non-human antibodies may be humanized by grafting only non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced. Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to zFGF5 protein or peptide, and selection of antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled zFGF5 protein or peptide).

Antibodies are defined to be specifically binding if they bind to a zFGF5 polypeptide with a binding affinity (K_a) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most

preferably 10^9 M^{-1} or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art (for example, by Scatchard analysis).

A variety of assays known to those skilled in the art can be utilized to detect antibodies which specifically bind to zFGF5 proteins or peptides. Exemplary assays are described in detail in Antibodies: A Laboratory Manual, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-precipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assay, inhibition or competition assay, and sandwich assay. In addition, antibodies can be screened for binding to wild-type versus mutant zFGF5 protein or peptide.

Antibodies to zFGF5 may be used for tagging cells that express zFGF5; to target another protein, small molecule or chemical to heart tissue; for isolating zFGF5 by affinity purification; for diagnostic assays for determining circulating levels of zFGF5 polypeptides; for detecting or quantitating soluble zFGF5 as marker of underlying pathology or disease; in analytical methods employing FACS; for screening expression libraries; for generating anti-idiotypic antibodies; and as neutralizing antibodies or as antagonists to block zFGF5 mediated proliferation *in vitro* and *in vivo*. Suitable direct tags or labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like; indirect tags or labels may feature use of biotin-avidin or other complement/anti-complement pairs as intermediates. Antibodies herein may also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications.

Molecules of the present invention can be used to identify and isolate receptors involved in cardiac myocyte, cardiac fibroblast, or cardiac progenitor cell proliferation. For example, proteins and peptides of the present invention can be immobilized on a column and membrane preparations run over the column (Immobilized Affinity Ligand Techniques, Hermanson et al., eds., Academic Press, San Diego, CA, 1992, pp.195-202). Proteins and peptides can also be radiolabeled (Methods in Enzymol., vol. 182, "Guide to Protein Purification", M. Deutscher, ed., Acad. Press, San Diego, 1990, 721-737) or photoaffinity labeled (Brunner et al., Ann. Rev. Biochem. 62:483-514, 1993 and Fedan et al., Biochem. Pharmacol. 33:1167-1180, 1984) and specific cell-surface proteins can be identified.

Antagonists will be useful for inhibiting the proliferative activities of zFGF5 molecules, in cell types such as cardiac cells, including myocytes, fibroblasts and endothelial cells, osteoblasts and chondrocytes. Genes encoding zFGF5 polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide display libraries are known in the art (Ladner et al., US Patent NO:5,223,409; Ladner et al., US Patent NO:4,946,778; Ladner et al., US Patent NO:5,403,484 and Ladner et al., US Patent NO:5,571,698) and random peptide display libraries and kits for screening such libraries are available commercially, for

instance from Clontech (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA) and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the zFGF5 sequences disclosed herein to identify proteins which bind to zFGF5. These "binding proteins" which interact with zFGF5 polypeptides may be used for tagging cells; for isolating homolog polypeptides by affinity purification; they can be directly or indirectly conjugated to drugs, toxins, radionuclides and the like. These binding proteins can also be used in analytical methods such as for screening expression libraries and neutralizing activity. The binding proteins can also be used for diagnostic assays for determining circulating levels of polypeptides; for detecting or quantitating soluble polypeptides as marker of underlying pathology or disease. These binding proteins can also act as zFGF5 "antagonists" to block zFGF5 binding and signal transduction *in vitro* and *in vivo*. These anti- zFGF5 binding proteins would be useful for inhibiting expression of genes which result in proliferation or differentiation. Such anti-zFGF5 binding proteins can be used for treatment, for example, in rhabdomyosarcoma, cardiac myxoma, bone cancers of osteoblast origin, and dwarfism, arthritis, ligament and cartilage repair, alone or combination with other therapies.

The molecules of the present invention will be useful for proliferation of cardiac tissue cells, such as cardiac myocytes, myoblasts or progenitors; skeletal myocytes or myoblasts and smooth muscle cells; chondrocytes; endothelial cells; adipocytes and osteoblasts *in vitro*. For example, molecules of the present invention are useful as components of defined cell culture media, and may be used alone or in combination with other cytokines and hormones to replace serum that is commonly used in cell culture. Molecules of the present

invention are particularly useful in specifically promoting the growth and/or development of myocytes in culture, and may also prove useful in the study of cardiac myocyte hyperplasia and regeneration.

The polypeptides, nucleic acid and/or antibodies of the present invention may be used in treatment of disorders associated with heart disease, i.e., myocardial infarction, coronary artery disease, congestive heart failure, hypertrophic cardiomyopathy, myocarditis, congenital heart defects and dilated cardiomyopathy. Molecules of the present invention may also be useful for limiting infarct size following a heart attack, promoting angiogenesis and wound healing following angioplasty or endarterectomy, to develop coronary collateral circulation, for revascularization in the eye, for complications related to poor circulation such as diabetic foot ulcers, for stroke, following coronary reperfusion using pharmacologic methods and other indications where angiogenesis is of benefit. Molecules of the present invention may be useful for improving cardiac function, either by inducing cardiac myocyte neogenesis and/or hyperplasia, by inducing coronary collateral formation, or by inducing remodelling of necrotic myocardial area.

An ischemic event is the disruption of blood flow to an organ, resulting in necrosis or infarct of the non-perfused region. Ischemia-reperfusion is the interruption of blood flow to an organ, such as the heart or brain, and subsequent restoration (often abrupt) of blood flow. While restoration of blood flow is essential to preserve functional tissue, the reperfusion itself is known to be deleterious. In fact, there is evidence that reperfusion of an ischemic area compromises endothelium-dependent vessel relaxation resulting in vasospasms, and in the heart compromised coronary vasodilation, that is not seen in an ischemic event without reperfusion (Cuevas et al., Growth Factors 15:29-40, 1997). Both ischemia and

reperfusion are important contributors to tissue necrosis, such as a myocardial infarct or stroke. The molecules of the present invention will have therapeutic value to reduce damage to the tissues caused by ischemia or ischemia-reperfusion events, particularly in the heart or brain.

Other therapeutic uses for the present invention include induction of skeletal muscle neogenesis and/or hyperplasia, kidney regeneration and/or for treatment of systemic and pulmonary hypertension.

zFGF5 induced coronary collateral development is measured in rabbits, dogs or pigs using models of chronic coronary occlusion (Landau et al., Amer. Heart J. 29:924-931, 1995; Sellke et al., Surgery 120(2):182-188, 1996 and Lazarous et al., 1996, ibid.) zFGF5 benefits for treating stroke is tested *in vivo* in rats utilizing bilateral carotid artery occlusion and measuring histological changes, as well as maze performance (Gage et al., Neurobiol. Aging 9:645-655, 1988). zFGF5 efficacy in hypertension is tested *in vivo* utilizing spontaneously hypertensive rats (SHR) for systemic hypertension (Marche et al., Clin. Exp. Pharmacol. Physiol. Suppl. 1:S114-116, 1995).

Molecules of the present invention can be used to target the delivery of agents or drugs to the heart. For example, the molecules of the present invention will be useful limiting expression to the heart, by virtue of the tissue specific expression directed by the zFGF5 promoter. For example, heart-specific expression can be achieved using a zFGF5-adenoviral discistronic construct (Rothmann et al., Gene Therapy 3:919-926, 1996). In addition, the zFGF5 polypeptides can be used to restrict other agents or drugs to heart tissue by linking zFGF5 polypeptides to another protein (Franz et al., Circ. Res. 73:629-638, 1993) by linking a first molecule that is comprised of a zFGF5 homolog polypeptide with a second

agent or drug to form a chimera. Proteins, for instance antibodies, can be used to form chimeras with zFGF5 molecules of the present invention (Narula et al., J. Nucl. Cardiol. 2:26-34, 1995). Examples of agents or drugs include, but are not limited to, bioactive-polypeptides, genes, toxins, radionuclides, small molecule pharmaceuticals and the like. Linking may be direct or indirect (e.g., liposomes), and may occur by recombinant means, chemical linkage, strong non-covalent interaction and the like.

Polynucleotides encoding zFGF5 polypeptides are useful within gene therapy applications where it is desired to increase or inhibit zFGF5 activity. If a mammal has a mutated or absent zFGF5 gene, the zFGF5 gene can be introduced into the cells of the mammal. In one embodiment, a gene encoding a zFGF5 polypeptide is introduced *in vivo* in a viral vector. Such vectors include an attenuated or defective DNA virus, such as, but not limited to, herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. A defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Examples of particular vectors include, but are not limited to, a defective herpes simplex virus 1 (HSV1) vector (Kaplitt et al., Molec. Cell. Neurosci. 2:320-30, 1991); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al., J. Clin. Invest. 90:626-30, 1992; and a defective adeno-associated virus vector (Samulski et al., J. Virol. 61:3096-101, 1987; Samulski et al., J. Virol. 63:3822-8, 1989).

In another embodiment, a zFGF5 gene can be introduced in a retroviral vector, e.g., as described in

Anderson et al., U.S. Patent No. 5,399,346; Mann et al. Cell 33:153, 1983; Temin et al., U.S. Patent No. 4,650,764; Temin et al., U.S. Patent No. 4,980,289; Markowitz et al., J. Virol. 62:1120, 1988; Temin et al., U.S. Patent No. 5,124,263; International Patent Publication No. WO 95/07358, published March 16, 1995 by Dougherty et al.; and Kuo et al., Blood 82:845, 1993. Alternatively, the vector can be introduced by lipofection *in vivo* using liposomes. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413-7, 1987; Mackey et al., Proc. Natl. Acad. Sci. USA 85:8027-31, 1988). The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. More particularly, directing transfection to particular cells represents one area of benefit. For instance, directing transfection to particular cell types in a tissue with cellular heterogeneity, such as the heart, brain, lungs or liver. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides (e.g., hormones or neurotransmitters), proteins such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

It is possible to remove the target cells from the body; to introduce the vector as a naked DNA plasmid; and then to re-implant the transformed cells into the body. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, e.g., transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun or use of a DNA vector transporter. See, e.g., Wu et al., J. Biol. Chem. 267:963-7, 1992; Wu et al., J. Biol. Chem. 263:14621-4, 1988.

Antisense methodology can be used to inhibit zFGF5 gene transcription, such as to inhibit cell proliferation in vivo. Polynucleotides that are complementary to a segment of a zFGF5-encoding polynucleotide (e.g., a polynucleotide as set froth in SEQ ID NO:1) are designed to bind to zFGF5-encoding mRNA and to inhibit translation of such mRNA. Such antisense polynucleotides are used to inhibit expression of zFGF5 polypeptide-encoding genes in cell culture or in a subject.

The present invention also provides reagents which will find use in diagnostic applications. For example, the zFGF5 gene, a probe comprising zFGF5 DNA or RNA or a subsequence thereof can be used to determine if the zFGF5 gene is present on chromosome 5 and if a mutation in the zFGF5 gene locus has occurred including, but not limited to, aneuploidy, gene copy number changes, insertions, deletions, restriction site changes and rearrangements. Such aberrations can be detected using polynucleotides of the present invention by employing molecular genetic techniques, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, and other genetic linkage analysis techniques known in the art (Sambrook et al., ibid.; Ausubel et. al., ibid.; Marian, Chest 108:255-65, 1995).

Mice engineered to express the zFGF5 gene, referred to as "transgenic mice," and mice that exhibit a complete absence of zFGF5 gene function, referred to as "knockout mice," may also be generated (Snouwaert et al., Science 257:1083, 1992; Lowell et al., Nature 366:740-42, 1993; Capecchi, M.R., Science 244: 1288-1292, 1989; Palmiter, R.D. et al. Annu Rev Genet. 20: 465-499, 1986). For example, transgenic mice that over-express zFGF5, either ubiquitously or under a tissue-specific or tissue-restricted promoter can be used to ask whether over-

expression causes a phenotype. For example, over-expression of a wild-type zFGF5 polypeptide, polypeptide fragment or a mutant thereof may alter normal cellular processes, resulting in a phenotype that identifies a tissue in which zFGF5 expression is functionally relevant and may indicate a therapeutic target for the zFGF5, its agonists or antagonists. For example, a preferred transgenic mouse to engineer is one that over-expresses the zFGF5 (approximately amino acid residue 28 to residue 207 of SEQ ID NO:2). Moreover, such over-expression may result in a phenotype that shows similarity with human diseases. Similarly, knockout zFGF5 mice can be used to determine where zFGF5 is absolutely required *in vivo*. The phenotype of knockout mice is predictive of the *in vivo* effects of a zFGF5 antagonist, such as those described herein, may have. These mice may be employed to study the zFGF5 gene and the protein encoded thereby in an *in vivo* system, and can be used as *in vivo* models for corresponding human diseases.

In one embodiment of the present invention, a composition comprising zFGF5 protein is used as a therapeutic agent to enhance osteoblast-mediated bone formation. The compositions and methods using the compositions of the invention may be applied to promote the repair of bone defects and deficiencies, such as those occurring in closed, open and non-union fractures; to promote bone healing in plastic surgery; to stimulate bone ingrowth into non-cemented prosthetic joints and dental implants; in the treatment of periodontal disease and defects; to increase bone formation during distraction osteogenesis; and in treatment of other skeletal disorders that may be treated by stimulation of osteoblastic activity, such as osteoporosis and arthritis. *De novo* bone formation provided by the methods of the present invention will have use in repair of congenital, trauma-

induced, oncologic resection of bone or healing bone following radiation-induced osteonecrosis (Hart et al, Cancer 37:2580-2585, 1976). The methods of the present invention may also find use in plastic surgery.

For pharmaceutical use, the proteins of the present invention are formulated for parenteral, particularly intravenous or subcutaneous, administration according to conventional methods. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. In general, pharmaceutical formulations will include a zFGF5 protein in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial surfaces, etc. Methods of formulation are well known in the art and are disclosed, for example, in Remington's Pharmaceutical Sciences, Gennaro, ed., Mack Publishing Co., Easton PA, 1990, which is incorporated herein by reference. Therapeutic doses will generally be in the range of 0.1 to 100 μg/kg of patient weight per day, preferably 0.5-20 μg/kg per day, with the exact dose determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Determination of dose is within the level of ordinary skill in the art. The proteins may be administered for acute treatment, over one week or less, often over a period of one to three days or may be used in chronic treatment, over several months or years.

In one embodiment, a therapeutically effective amount of zFGF5 is an amount sufficient to produce a clinically significant change in myocyte proliferation, heart function, bone formation or increases in specific cell types associated with mesenchymal stem cells and

progenitors for myocytes, osteoblasts and chondrocytes. In particular, a clinically significant improvement in cardiac performance may be an increase in the number of myocytes or myocyte progenitor cells. Improvements in cardiac performance can be determined by methods well known and accepted by clinicians and those skilled in the art. Such determinations include, but are not limited to, measuring the left ventricular ejection fraction, prior to, and after administration of zFGF5 molecules, and determining at least a 5% increase, preferably 10% or more, in the total ejection fraction, increases in -dP/dt or +dP/dt, greater exercise tolerance, a decrease in vascular resistance, and increased blood flow to the heart. A reduction in symptoms may also be indication of a significant improvement in cardiac performance, and include, for example, reduction in angina pectoris, breathlessness, leg swelling, heart or respiratory rates, edema, fatigue and weakness.

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

<u>Example 1</u>

<u>Extension of EST Sequence</u>

Scanning of a translated DNA database using a query for growth factors resulted in identification of an expressed sequence tag (EST) sequence found to be a novel member of the FGF family, and designated zFGF5.

Oligonucleotide primers ZC11676 (SEQ ID NO: 3) and ZC11677 (SEQ ID NO: 4) were designed from the sequence of an expressed sequence tag (EST). The primers were used for priming internally within the EST, and when PCR was performed using MARATHON READY cDNA (Clontech, Palo Alto,

CA) from adult heart tissue as template in polymerase chain reaction (PCR).

The conditions used for PCR were 1 cycle at 94°C for 90 seconds, 35 cycles at 94°C for 15 seconds; 68°C for 1 minute; followed by 1 cycle for 10 minutes at 72°C and 4°C incubation period. The PCR reaction recreated 160 bp of the EST sequence, and confirmed that EST sequence was correct.

Other libraries that could be amplified with the oligonucleotide primers included skeletal muscle, lung, stomach, small intestine and thyroid.

Example 2
Tissue Distribution

Northerns were performed using Human Multiple Tissue Blots from Clontech (Palo Alto, CA). The 160 bp DNA fragment described in Example 1 was electrophoresed on a 1% agarose gel, the fragment was electroeluted, and then radioactively labeled using a random priming MEGAPRIME DNA labeling system (Amersham, Arlington Heights, IL) according to the manufacturer's specifications. The probe was purified using a NUCTRAP push column (Stratagene Cloning Systems, La Jolla, CA). EXPRESSHYB (Clontech, Palo Alto, CA) solution was used for prehybridization and as a hybridizing solution for the Northern blots. Hybridization took place overnight at 68°C, and the blots were then washed in 2X SSC and 0.05% SDS at RT, followed by a wash in 0.1X SSC and 0.1% SDS at 50°C. A single band was observed at approximately 2.0 kb. Signal intensity was highest for adult heart with relatively less intense signals in skeletal muscle and stomach. Dot blots were probed essentially as described above, confirming that expression for human zFGF5 was highest in heart tissue followed by lung and skeletal muscle.

Example 3
Assay for *In Vitro* Activity of zFGF5

A.

The mitogenic activity of zFGF5 is assayed using cell lines and cells from a primary culture. Conditioned medium from cells expressing the recombinant protein and/or purified protein is added to cultures of the following cell lines: NIH 3T3 fibroblast (ATCC No. CRL-1658), CHH-1 chum heart cells (ATCC No. CRL-1680), H9c2 rat heart myoblasts (ATCC No. CRL-1446), Shionogi mammary carcinoma cells (Tanaka et al., 1992, ibid.) and LNCaP.FGC adenocarcinoma cells. Freshly isolated cells useful for testing the proliferative activity of zFGF5 include: cardiac fibroblasts, cardiac myocytes, skeletal myocytes and human umbilical vein endothelial cells.

Mitogenic activity is assayed by measurement of ^{3}H-thymidine incorporation based on the method of Raines and Ross (Meth. Enzymology 109:749-773, 1985). Briefly, quiescent cells are plated cells at a density of 3×10^4 cells/ml in an appropriate medium. A typical growth medium is Dulbecco's Growth Medium (GIBCO-BRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS). The cells are cultured in 96-well plates and allowed to grow for 3-4 days. The growth medium is removed, and 180 µl of DFC (Table 5) containing 0.1% FCS is added per well. Half the wells have zFGF5 protein added to them and the other half are a negative control, without zFGF5. The cells are incubated for up to 3 days at 37°C in 5% CO_2, and the medium is removed. One hundred microliters of DFC containing 0.1% FCS and 2 µCi/ml ^{3}H-thymidine is added to each well, and the plates are incubated an additional 1-24 hours at 37°C . The medium is aspirated off, and 150 µl of trypsin is added to each well. The plates are incubated at 37°C until the cells detached (at least 10 minutes). The detached cells are harvested onto filters using an LKB Wallac 1295-001 Cell Harvester (LKB Wallac, Pharmacia,

Gaithersburg, MD). The filters are dried by heating in a microwave oven for 10 minutes and counted in an LKB Betaplate 1250 scintillation counter (LKB Wallac) as described by the supplier.

TABLE 5

250 ml Dulbecco's Modified Eagle's Medium (DMEM, Gibco-BRL)
250 ml Ham's F12 medium (Gibco-BRL)
0.29 mg/ml L-glutamine (Sigma, St. Louis, MO)
1 mM sodium pyruvate (Sigma, St. Louis, MO)
25 mM Hepes (Sigma, St. Louis, MO)
10 μg/ml fetuin (Aldrich, Milwaukee, WI)
50 μg/ml insulin (Gibco-BRL)
3 ng/ml selenium (Aldrich, Milwaukee, WI)
20 μg/ml transferrin (JRH, Lenexa, KS)

B.

Hearts were isolated from 1 day old neonatal mice and then disrupted by repeat collagenase digestions, following the protocol of Brand et al., (J. Biol. Chem. 268:11500-11503, 1993). Individual myocytes were isolated over a Percoll gradient, and 2 ml were plated in 6 well tissue culture dishes at 0.5 X 10^6 cells/ml. Three days later the wells were washed 3 times with PBS without calcium or magnesium, and refed with 1 ml serum free medium (Table 6). The wells were inoculated with 10^{11} particles AdCMV-zFGF5 per well or AdCMV-GFP (green fluorescent protein) as a control, and incubated at 37°C for 8 hours. The wells were then washed again 3 times with PBS without calcium or magnesium, and then refed with 2 mls serum free media.

Within 48 hours after inoculation with the AdCMV-zFGF5, the cultured myocytes have ceased to beat and have undergone a morphologic alteration, while the wells inoculated with the AdCMV-GFP continued to beat

spontaneously and are unaffected morphologically by the inoculation. Wells inoculated with AdCMV-zFGF5 also contained, after 48, hours, a confluent layer of viable, non-adherent cells, without any loss in confluence of the adherent myocyte layers, indicating the proliferative activity of the adCMV-zFGF5 on cultured murine myocytes.

<u>Table 6</u>

DMEM

Ham's Nutrient Mixture F12 (Gibco-BRL; 1:1 mixture with DMEM)

17 mM $NaHCO_3$ (Sigma)

2 mM L-glutamine (Sigma)

1% PSN (Sigma)

1 µg/ml insulin

5 µg/ml transferrin

1 nM LiCl (Sigma)

1 nM selenium

25 µg/ml ascorbic acid(Sigma)

1 nM thyroxine (Sigma)

C.

zFGF5 fused to a maltose binding protein (MBP), as described in Example 9A and purified as described in Example 10, was added to myocytes (Example 3B) at a concentration of 0.1 ng/ml. MBP-zFGF5 was shown to stimulate proliferation of myocytes, as well.

<u>Example 4</u>

<u>Assay for *Ex Vivo* Activity of zFGF5</u>

Cardiac mitogenesis is measured *ex vivo* by removing entire hearts from neonatal or 8-week old mice or rats. The excised heart is placed in Joklik's (Sigma, St. Louis, MO) or Dulbecco's medium at 37°C, 5% CO_2 for 4-24 hours. During the incubation period zFGF5 polypeptide is added at a concentration range of 1 pg/ml to 100 µg/ml. Negative controls are using buffer only. ^{3}H-thymidine is added and the samples are incubated for 1-4 hours, after

which the heart is sectioned and mitogenesis is determined by autoradiography. Sections are used for histomorphometry to determine the nuclei/cytoplasmic volume (McLaughlin, Am. J. Physiol. 271:R122-R129, 1996.)

Alternatively, the heart was lyophilized and resuspended in 1 ml 0.1 N NaOH. The DNA was precipitated using ice cold 10% trichloroacetic acid (TCA). The supernatant was added to 9 ml scintillation fluid to measure non-specific ^{3}H-thymidine incorporation. The resulting pellet was resuspended in 1 ml BTS-450 tissue solubilizer (Beckman, Fullerton, CA) and added to 9 ml of scintillation fluid to measure specific DNA incorporation of ^{3}H-thymidine.

Left and right ventricles were isolated from 1 day old CD-1 mice (Jackson Labs, Bar Harbor, ME), and incubated for 4 hours with 3 ng/ml zFGF5Hep2 (n=13; see Example 10) or control (n=10). ^{3}H-thymidine was added for 1 hour. The ventricles were washed several times and then homogenized in 1 ml Joklik's medium. The resulting homogenate was added to 9 ml scintillation cocktail and analyzed for total ^{3}H-thymidine uptake and DNA incorporation.

zFGF5-Hep2 increased ^{3}H-thymidine uptake and incorporation in DNA 2.068 $\pm$ 0.489 fold over control, indicating that zFGF5 is mitogenic for a cardiac cell.

Example 5

Assay for *In Vivo* Activity of zFGF5

The proliferative effects of zFGF5 are assayed *in vivo* using two-week old neonatal rats and/or two-month old adult rats. The rats are injected intraperiocardially either acutely or chronically.

A.

Neonatal rats are treated with zFGF5 for 1 to 14 days over a dose range of 50 ng/day to 100 µg/day. After treatment, the effects of zFGF5 versus the sham-treated

animals is evaluated by measuring increased cardiac weight, improved *in vivo* and *ex vivo* left ventricular function, and by increased cardiac nuclear to cytosolic volume fractions, that are determined histomorphometrically.

B.

Rats with cardiomyopathy induced by chronic catecholamine infusion, by coronary ligation or for models of cardiomyopathy such as the Syrian Cardiomyopathic hamster (Sole et al., Amer. J. Cardiol. 62(11):20G-24G, 1988) are also used to evaluate the effects of zFGF5 on cardiac function and tissue.

To induce cardiomyopathy using catecholamine, 7-8 week old rats are infused continuously with epinephrine for 2 weeks via osmotic minipumps implanted subcutaneously between their shoulder blades. The epinephrine infusion results in an increase in the left ventricular fibrotic lesion score from 0.005 ± 0.005 to 2.11 ± 0.18, scale from 0-3); increased left ventricular myocyte cell width from 17.36 ± 0.46 µm to 23.05 ± 0.62 µm; and negligible left ventricular papillary muscle contractile responses to isoproterenol (0.2 vs 1.1 grams tension compared to saline-infused rats. After the two week treatment period, the rats are injected intraperiocardially daily with either vehicle, zFGF5, bFGF, IGF-I or IGF-II for up to 14 days. The rats are sacrificed and histomorphometry and histocytochemistry are performed.

Rats, treated as described above, are also evaluated at the end of the cathecholamine treatment, and again after growth factor treatment, where cardiac regeneration is measured as decreased left ventricular fibrotic lesion scores, reduced myocyte cell width and increased left ventricular papillary contractile responses to isoproterenol.

Example 6

Chromosomal Mapping of zFGF5

ZFGF5 was mapped to chromosome 5 using the commercially available version of the Whitehead Institute/MIT Center for Genome Research's "GeneBridge 4 Radiation Hybrid Panel" (Research Genetics, Inc., Huntsville, AL). The GeneBridge 4 Radiation Hybrid Panel contains DNAs suitable for PCR use from each of 93 radiation hybrid clones, plus two control DNAs (the HFL donor and the A23 recipient). A publicly available WWW server (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl) allows mapping relative to the Whitehead Institute/MIT Center for Genome Research's radiation hybrid map of the human genome (the "WICGR" radiation hybrid map) which was constructed with the GeneBridge 4 Radiation Hybrid Panel.

For the mapping of zFGF5 with the "GeneBridge 4 RH Panel", 25 µl reactions were set up in a 96-well microtiter plate (Stratagene, La Jolla, CA) and used for PCR in a "RoboCycler Gradient 96" thermal cycler (Stratagene). Each of the 95 PCR reactions consisted of 2.5 µl 50X "Advantage KlenTaq Polymerase Mix" (Clontech), 2 µl dNTPs mix (2.5 mM each; Perkin-Elmer, Foster City, CA), 1.25 µl sense primer, ZC11677 (SEQ ID NO: 4) 1.25 µl antisense primer, ZC12053 (SEQ ID NO: 5).

2.5 µl "RediLoad" (Research Genetics, Inc), 0.5 µl "Advantage KlenTaq Polymerase Mix" (Clontech Laboratories, Inc.), 25 ng of DNA from an individual hybrid clone or control and ddH2O for a total volume of 25 µl. The reactions were overlaid with an equal amount of mineral oil and sealed. The PCR cycler conditions were as follows: an initial 1 cycle of 4 minutes at 94°C, 35 cycles of 1 minute at 94°C, 1.5 minute annealing at 66°C and 1.5 minute extension at 72°C, followed by a final 1 cycle extension of 7 minutes at 72°C. The reactions were

separated by electrophoresis on a 3% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME).

The results showed that zFGF5 maps 541.12 cR from the top of the human chromosome 5 linkage group on the WICGR radiation hybrid map. Relative to the centromere, its nearest proximal marker was WI-16922 and its nearest distal marker was WI-14692. The use of surrounding CHLC map markers also helped position zFGF5 in the 5q34-q35 region on the CHLC chromosome 5 version v8c7 integrated marker map (The Cooperative Human Linkage Center, WWW server-http://www.chlc.org/ChlcIntegratedMaps.html).

Example 7
zFGF5 Effects on Bone

A.

An adenovirus vector containing the cDNA for zFGF5 was constructed using methods described by Becker et al. (Methods in Cell Biology 43:161-189, 1994). Briefly, the cDNA for zFGF5 (as shown in SEQ ID NO: 1) was cloned as a Xba I-Sal I fragment into pACCMV (Gluzman et al., In Eucaryotic Viral Vectors, Gluzman (eds.) pp.187-192, Cold Spring Harbor Press, Cold Springs Harbor NY, 1982). The pACCMV vector contains part of the adenovirus 5 genome, the CMV promoter and an SV40 terminator sequence. The plasmid containing the vector and cDNA insert was cotransfected with a plasmid containing the the adenovirus 5 genome, designated pJM17, (McGrory et al., Virology 163:614-617, 1988) into 293 cells (ATCC No. CRL-1573; American Type Culture Collection, Rockville, MD), leading to a recombination event and the production of a recombinant adenovirus containing zFGF5, designated AdCMV-zFGF5. The presence of the zFGF5 cDNA was confirmed by PCR.

The adenovirus vector AdCMV-zFGF5 was used for gene tranfer *in vivo* by intravenous injection of between 1 X 10^{11} and 5 x 10^{11} particles/mouse. It has been shown that after intravenous injection, the majority of the virus targets the liver and very efficiently transduces hepatocytes (Herz et al., Proc. Natl. Acad. Sci. USA 90:2812-2816, 1993). It has been demonstrated that the cells produce the protein encoded by the cDNA, and in the case of secreted proteins, secret them into the circulation. High levels of expression and physiological effects have been demonstrated (Ohwada et al., Blood 88:768-774, 1996; Stevenson et al., Arteriosclerosis, Thrombosis and Vascular Biology, 15:479-484, 1995; Setoguchi et al., Blood 84:2946-2953, 1994; and Sakamoto et al., Proc. Natl. Acad. Sci. USA 91:12368-12372, 1994).

Six week old CD-1 mice (Jackson Labs, Bar Harbor, ME) were treated with adenovirus containing no cDNA insert (AdCMV-null) or AdCMV-zFGF5 either IV through the tail vein or intrapericardially (IPC). A total of 5 X 10^{11} viral particles/100 µl/mouse were given. 14 days after injection, the animals were sacrificed, and tibias and femurs were removed without being separated to examine any potential inflammatory response. The bones were fixed in 10% neutral buffered formalin and processed. They were decalcified in 5% formic acid with 10% sodium citrate, washed in water, dehydrated in a series of 70%-100% ethanol, cleared in xylene and embedded in paraffin. The specimens were cut longitudinally through both tibial and femoral metaphyses and stained with hematoxylin and eosin for identification of bone cells. Osteoblasts were identified by central negative Golgi area and eccentric nucleus, while osteoclasts were identified by multinucleation, non-uniform shape and the Howship's lacunae associated with these resorbing cells.

For bone histomorphometry, femur samples were chosen. Cancellous bone volume was not measured due to

variation in the sampling site (i.e., femur samples were not sectioned exactly at the same plane). Three bone parameters were evaluated for histomorphometric changes.

1. Number of endosteal osteoblasts: measured along the endosteal surface of cancellous bone at 180 X magnification in an area 1.22 mm proximal to the growth plate.

2. Number of endosteal osteoclasts: measured along the endosteal surface of cancellous bone at 180 X magnification in an area 1.22 mm proximal to the growth plate.

3. Growth plate width: measured every 72 μm at 90 X magnification across the entire growth plate except at the peripheral ends to determine the growth plate activity.

Analyses of the data (mean ± SD, n=4-7/group) demonstrated the following:

1. There appeared to be no detectable inflammatory response at the joint between tibia and femur.

2. AdCMV-zFGF5 given IV or IPC in mice significantly increased osteogenic activity in the distal femural metaphysis, when examined at 2 weeks. This stimulation of osteogenic activity was indicated by:

a) significant increases in the number of endosteal osteoblasts in the cancellous bone of distal femurs following IV infusion or IPC injection of AdCMV-zFGF5, 530% and 263%, respectively, when compared with their relative vector only controls; and

b) the observation of increased osteogenic tissues on the bone surface, suggesting increased differentiation of bone marrow stromal cells toward the osteoblast lineage.

3. The number of endosteal osteoclasts was not significantly affected by IV or IPC administration of

AdCMV-zFGF5, when compared with their relative vector only controls.

4. The growth plate width was significantly decreased by IV infusion, but not IPC injection, of AdCMV-zFGF5, suggesting depressed growth plate activity following IV infusion. The differential effects of AdCMV-zFGF5 administrations have not been elucidated.

These results suggest that zFGF5 is a strong mitogen for stimulation of osteoblast proliferation and that zFGF5 has the capacity to induce new bone formation.

B.

Using essentially the same procedures described above in 7.A. QCT was done on female CD-1 (Jackson Labs) that were injected with 1 $x10^{11}$ particles AdCMV-zFGF5 per mouse. The mice were sacrificed 30 days after injection and heart/tibial length ratios were increased compared to controls (injected with empty adenorvirus or saline). There were no differences between the groups in tibial lengths to account for the change, nor were there differences in any other organ weights among the groups. Thus, the indication is that zFGF5 adenovirus selectively increases total bone density, trabecular bone density, and cortical thickness in the femur, as measured by QCT.

Example 8
Effects of zFGF5 on Heart

As described in 7.B. CD-1 mice were given a single IV injection of AdCMV-zFGF5, sacrificed after four weeks, and the heart/tibial length ratios were found to be increased compared to empty adenovirus or saline treated mice. The results showed that there were no differences between the groups in tibial lengths to account for this change, nor were there differences in any other organ weights among the groups. This result suggests that

AdCMV-zFGF5 selectively increased cardiac growth, when administered as an IV adenoviral construct.

Example 9

Expression of zFGF5

A. Construction of zFGF5-Encoding Plasmids

zFGF5, a fibroblast growth factor homolog, was expressed in *E. coli* using the MBP (maltose binding protein) fusion system from New England Biolabs (NEB; Beverly, MA). In this system, the zFGF5 cDNA was attached to the 3' end of the malE gene to form an MBP-zFGF5 fusion protein. Fusion protein expression was driven by the tac promoter; expression is "off" until the promoter is induced by addition of 1 mmol IPTG (isopropyl b-thiogalactosylpyranoside). Three variations of this fusion protein were made, differing only in their cleavage site for liberating zFGF5 from MBP. One construct had a thrombin cleavage site engineered between the MBP and zFGF5 domains. The second construct had a Factor Xa cleavage site, instead of a thrombin cleavage site. The third construct had an enterokinase cleavage site, instead of the thrombin cleavage site.

The constructs were built as in-frame fusions with MBP in accordance with the Multiple Cloning Site (MCS) of the pMAL-c2 vector (NEB), and according to the manufacturer's specifications. zFGF5 was amplified via PCR using primers which introduced convenient cloning sites, as well as cleavage sites using the following oligonucleotide primers: 1) for the thrombin construct: zc12652 (SEQ ID NO: 7) and zc12631 (SEQ ID NO: 8); 2) for the Factor Xa construct: zc15290 (SEQ ID NO: 9) and zc12631 (SEQ ID NO: 8); and 3) for the enterokinase construct: zc15270 (SEQ ID NO: 10) and zc12631 (SEQ ID NO: 8). In each case, the native zFGF5 signal sequence was not amplified; the zFGF5 as expressed begins at amino acid

residue 26 of SEQ ID NO: 2 (Val was changed to an Ala). The thrombin construct was built by inserting an Xba I-Sal I zFGF5 fragment into the Xba I-Sal I sites of pMAL-c2. The Factor Xa construct was built by inserting a blunt-Sal I fragment into the Xmn I-Sal I sites of the MCS. The enterokinase construct was built by inserting an Xba I-Sal I fragment into the Xba-Sal I sites of pMAL-c2. Once the constructs were built, they were transformed into a variety of *E. coli* host strains and analyzed for high-level expression. The thrombin construct (designated pSDH90.5) was transfected into DH10B cells (GIBCO-BRL), while both the Factor Xa construct (designated pSDH117.3) and the enterokinase construct (designated pSDH116.3) were transfected into TOP10 cells (Invitrogen, San Diego, CA). All three MBP fusions are about 63 kD (43 kD in the MBP domain, and approximately 20 kD in the zFGF5 domain).

B. Homologous Recombination/ zFGF5

Expression of zFGF5 in *Pichia methanolica* utilizes the expression system described in co-assigned PCT publication WO97/17450, incorporated herein by reference. An expression plasmid containing all or part of a polynucleotide encoding zFGF5 is constructed via homologous recombination. The expression vector is built from pCZR204, which contains the AUG1 promoter, followed by the αFpp leader sequence, followed by an amino-terminal peptide tag, a blunt-ended SmaI restriction site, a carboxy-terminal peptide tag, a translational STOP codon, followed by the AUG1 terminator, the ADE2 selectable marker, and finally the AUG1 3' untranslated region. Also included in this vector are the URA3 and CEN-ARS sequences required for selection and replication in *S. cerevisisiae*, and the Amp^R and colE1 ori sequences required for selection and replication in *E. coli.* The zFGF5 sequence inserted into this vector begins at residue 27 (Ala) of the zFGF amino acid sequence.

To construct pSDH114, a plasmid for expression of zFGF5 in *P. methanolica*, the following DNA fragments were transformed into *S. cerevisisae*: 100 ng of the 'acceptor vector' pCZR204 that has been digested with SmaI; 1 μg of an XbaI-SalI restriction fragment liberated from pSDH90.5 and encompassing zFGF5 coding sequence.; 1 μg of a synthetic, PCR-generated, double-stranded linker segment that spans 70 base pairs of the aFpp coding sequence on one end and joins it to the 70 base pairs of the amino-terminus coding sequence from the mature zFGF5 sequence on the other was generated from the four oligonucleotides zc13497 (SEQ ID NO: 11); zc15131 (SEQ ID NO: 12); zc15132; (SEQ ID NO: 18); zc15134 (SEQ ID NO: 13), of which the sense strand of a double stranded sequence is shown in SEQ ID NO: 19 (5' linker sequence (aFpp -> zFGF5 N-terminus)) and 1 μg of of a synthetic, PCR-generated, double-stranded linker segment that spans 70 base pairs of carboxy-terminus coding sequence from zFGF5 on one end with 70 base pairs of AUG1 terminator sequence was generated from the four oligonucleotides 13529 (SEQ ID NO: 14); zc13525 (SEQ ID NO: 15) zc13526 (SEQ ID NO: 16); zc13528 (SEQ ID NO: 17) of which the sense strand of a double stranded sense is shown in the SEQ ID NO: 20 (3' linker sequence (zFGF5 C-terminus -> *AUG1* terminator)). Ura^+ colonies were selected, and DNA from the resulting yeast colonies was extracted and transformed into *E. coli*. Individual clones harboring the correct expression construct were identified by PCR screening with oligonucleotides zc13497 (SEQ ID NO: 11) and zc13528 (SEQ ID NO: 12) followed by restriction digestion to verify the presence of the zFGF5 insert and DNA sequencing to confirm the desired DNA sequences had been enjoined with one another. Larger scale plasmid DNA is isolated for one of the correct clones, and the DNA is digested with Sfi I to liberate the *Pichia*-zFGF5 expression cassette from the vector backbone. The Sfi I-cut DNA is then transformed

into a *Pichia methanolica* expression host, designated PMAD16, and plated on ADE D plates for selection. A variety of clones are picked and screened via Western blot for high-level zFGF5 expression.

More specifically, for small-scale protein production (e.g., plate or shake flask production), *P. methanolica* transformants that carry an expression cassette comprising a methanol-regulated promoter (such as the *AUG1* promoter) are grown in the presence of methanol and the absence of interfering amounts of other carbon sources (e.g., glucose). For small-scale experiments, including preliminary screening of expression levels, transformants may be grown at 30°C on solid media containing, for example, 20 g/L Bacto-agar (Difco), 6.7 g/L yeast nitrogen base without amino acids (Difco), 10 g/L methanol, 0.4 mg/L biotin, and 0.56 g/L of -Ade -Thr -Trp powder. Because methanol is a volatile carbon source it is readily lost on prolonged incubation. A continuous supply of methanol can be provided by placing a solution of 50% methanol in water in the lids of inverted plates, whereby the methanol is transferred to the growing cells by evaporative transfer. In general, not more than 1 ml of methanol is used per 100-mm plate. Slightly larger scale experiments can be carried out using cultures grown in shake flasks. In a typical procedure, cells are cultivated for two days on minimal methanol plates as disclosed above at 30°C, then colonies are used to inoculate a small volume of minimal methanol media (6.7 g/L yeast nitrogen base without amino acids, 10 g/L methanol, 0.4 mg/L biotin) at a cell density of about 1 x 10^6 cells/ml. Cells are grown at 30°C. Cells growing on methanol have a high oxygen requirement, necessitating vigorous shaking during cultivation. Methanol is replenished daily (typically 1/100 volume of 50% methanol per day).

For production scale culturing, fresh cultures of high producer clones are prepared in shake flasks. The resulting cultures are then used to inoculate culture medium in a fermenter. Typically, a 500 ml culture in YEPD grown at 30°C for 1-2 days with vigorous agitation is used to inoculate a 5-liter fermenter. The cells are grown in a suitable medium containing salts, glucose, biotin, and trace elements at 28°C, pH 5.0, and >30% dissolved O_2. After the initial charge of glucose is consumed (as indicated by a decrease in oxygen consumption), a glucose/methanol feed is delivered into the vessel to induce production of the protein of interest. Because large-scale fermentation is carried out under conditions of limiting carbon, the presence of glucose in the feed does not repress the methanol-inducible promoter.

Example 10

Purification of zFGF5

E.coli fermentation medium was obtained from a strain expressing zfGF5 as a Maltose Binding protein fusion (pSDH90.5, as described above). The MBPzFGF5 fusion was solubilized during sonication or French press rupture, using a buffer containing 20 mM Hepes, 0.4 M Nacl, 0.01 M EDTA, 10 mM DTT, at pH 7.4. The extraction buffer also included 5 µg/ml quantities of Pepstatin, Leupeptin, Aprotinin, Bestatin. Phenyl methyl sulfonylfluoride (PMSF) was also included at a final concentration of 0.5 mM.

The extract was spun at 18,000 x g for 30 minutes at 4°C. The resulting supernatent was processed on an Amylose resin (Pharmacia LKB Biotechnology, Piscataway, NJ) which binds the MBP domain of the fusion. Upon washing the column, the bound MBPzFGF5 fusion was

eluted in the same buffer as extraction buffer without DTT and protease inhibitors but containing 10 mM Maltose.

The eluted pool of MBPzFGF5 was treated with 1:100 (w/w) Bovine thrombin to MBPzFGF5 fusion. The cleavage reaction was allowed to proceed for 6 to 8 hours at room temperature, after which the reaction mixture was passed over a bed of Benzamidine sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) to remove the thrombin, using the same elution buffer as described above for Amylose affinity chromatography.

The passed fraction, containing the cleaved product zFGF5 and free MBP domain were applied to a Toso Haas Heparin affinity matrix (Toso Haas, Montgomeryville, PA) equilibrated in 0.5 M NaCl, 20 mM Hepes, 0.01 M EDTA at pH 7.4. The MBP and zFGF5 both bound to heparin under these conditions. The bound proteins were eluted with a 2 to 3 column volume gradient formed between 0.5M NaCl and 2.0 M NaCl in column buffer.

The MBP eluted early, at about 0.7 M NaCl, and the cleaved zFGF5 eluted at about 1.3 M NaCl. The pooled zFGF5 fractions were passed through the amylose step once again to remove any residual MBPzfgf5 that is a minor contaminant. The purified material was designated zFGF5-Hep2, and shows a single highly pure species at ~20 kDa on reducing SDS-PAGE analysis.

Amino acid N-terminal sequencing yielded the native N-Terminal sequence but Mass Spectrophotometry data revealed molecular masses indicating that the C-Terminus must be truncated at residue 196 (Lys) of SEQ ID NO: 2, where a "dibasic site" is present.

zFGF5 protein was very stable in 1.3 M NaCl. Upon dialysis into PBS, the zFGF5 aggregated and left the solution phase. Therefore, formulations that include heparin and other "polyanions" may be used to prevent the aggregation of pure zFGF5.

Example 11

Production of Antibodies

Antibodies for ZFGF5 were produced, using standard techniques known in the art and described previously, by immunizing guinea pigs, rabbits and mice with peptides QTRARDDVSRKQLRLYC (SEQ ID NO: 2 amino acid residue 40 to residue 56), designated zFGF-1; YTTVTKRSRRIRPTHRAC (SEQ ID NO: 2 amino acid residue 191 to residue 207, with an additional Cys at the C-terminus), designated zFGF5 or the full-length zFGF5 polypeptide as shown in SEQ ID NO: 2, plus the MPB fusion protein, and designated MBP-FGF5. Peptides were conjugated through Cys residues using Maleimide-activated KLH (Pierce Chemical Co., Rockford, IL).

Table 7 is a description of the animals, immunization levels and antibody separations.

Table 7

Peptide or Protein	animal	immun. level	Ab produced
ZFGF5-1	G.P.	50ug/animal initial 25ug/animal boost	Affinity purified and IgG fractionated.
	Rabbit	100ug/animal initial 50ug/animal boost	Affinity purified and IgG fractionated.
ZFGF5-2	G.P.	50ug/animal initial 25ug/animal boost	Affinity purified and IgG fractionated.
	Rabbit	100ug/animal initial 50ug/animal boost	Affinity purified. and IgG fractionated.
ZFGF5-MBP	Mouse	20ug/animal initial 10ug/animal boost	
	Rabbit	200ug/animal initial 100ug/animal boost	Affinity purified

Example 12

Effects of zFGF5 on ob/ob Mice

The effects of zFGF5 on adipocytes and fat metabolism were examined using female ob/ob mice (C57B1/6J, Jackson Labs, Bar Harbor, ME). The mice are obese, insulin resistant and have "fatty bone". The mice were weighed and all were found to be the same weight, and were injected IV with 10^{11} particles per mouse of AdCMVzFGF5 or either saline or Ad5CMV-GFP for controls, as described in Example 7. 17 days after injection, the control mice injected with Ad5CMV-GFP had gained 5.342 $\pm$ 0.5 grams of body weight compared to the day of injection, while the AdCMVzFGF5 treated mice lost 3.183 $\pm$0.743 grams of body weight.

Example 13

A. Cloning of Mouse zFGF5

A cDNA for the mouse ortholog of zFGF5 was isolated from a mouse embryo library. Oligonucleotide primers were designed from the full length human zFGF5 sequence (ZC17578 and ZC17579, SEQ ID NOS: 37 and 38, respectively). A PCR reaction was done using 2 µl of library as template and ExTaq polymerase (PanVera, Madison, WI) under the following conditions 1 cycle at 94°C for 15 seconds; 35 cycles at 94°C for 15 seconds, 60°C for 20 seconds, 72°C for 30 seconds; and 1 cycle at 72°C for 10 minutes. The reaction mixture was incubated a 4°C overnight. After the first reaction was screened, no positive clones were identified and the procedure was repeated until a positive clone was identified. The positive clones were identified by transforming ElectroMAZ DH10B cells (GibcoBRL) with 1 µl of reaction mixture at 2.3 kV. The cells were plated on culture plates containing ampicillin and methicillin and incubated at room temperature for 3 days.

A DNA fragment obtained by PCR as described above was radiolabeled using a Multiprime DNA Labeling System (Amersham) and used as a probe for filters lifted from culture plates. The filter lifts were hybridized overnight at 65°C in EXPRESS HYB (Clontech). After hybridiziation, the filters were washed in buffer of 0.25X SSC, 0.25% SDS, 1 mM EDTA at 65°C, 6 times.

Positive clones were identified and cDNA inserts were screened. The clones identified had truncations at the 5' end, complete at the 3' ends and included 3' UTR. One clone, designated LC 7-2 had the longest 5' end when compared to the human zFGF5 sequence. Sequence analysis verified that approximately 52 bp of 5' sequence were missing and that this sequence was in the signal sequence and that the entire nucleotide sequence encoding the mature polypeptide was intact.

B. Northern Analysis

Northern analyses were performed using Mouse Multiple Tissue Blots from Clontech (Palo Alto, CA), mouse heart blots (prepared at ZymoGenetics, Inc.) and mouse dot blots (Clontech). Using oligonucleotides ZC17579 (SEQ ID NO: 29) and ZC17578 (SEQ ID NO: 40) and the mouse zFGF5 as a template, a probe was generated. The DNA probe was radioactively labeled using a random priming MEGAPRIME DNA labeling system (Amersham, Arlington Heights, IL) according to the manufacturer's specifications. The probe was purified using a NUCTRAP push column (Stratagene Cloning Systems, La Jolla, CA). EXPRESSHYB (Clontech, Palo Alto, CA) solution was used for prehybridization and as a hybridrizing solution for the Northern blots. Hybridization took place overnight at 68°C, and the blots were then washed in 2X SSC and 0.05% SDS at RT, followed by a wash in 0.1X SSC and 0.1% SDS at 50°C. Multiple bands were observed at with predominate bands at approximately 0.6-0.8 kb, 1.2 kb and 2.2-2.4 kb

bands depending on the blot used. Signal intensity was highest for spleen with slightly lower intensity signals in heart, lung, liver, skeletal muscle, kidney and testis. Mouse dot blots with the same probe were positive only for spleen and day 17 mouse embryo. Mouse heart mRNA northerns were probed and results were positive for C57 Black, CD1, neonatal heart, and day 16 and day 20 embryo, with strongest signal present in the day 16 embryo. BALB C mouse heart did not have a signal present.

Because the results in the mouse tissue did not directly correlate with results seen in the human tissue, a new probe was designed. The new probe was designed specifically to exclude the possibility that any members of the FGF family other than zFGF5 were positive by Northern analysis. The probe was prepared using PCR with oligonucleotides ZC195687 (SEQ ID NO: 41) and ZC19633 (SEQ ID NO: 42) and template DNA from the mouse cDNA of zFGF5. The reactions were essentially the same as described above. The mouse heart blot was positive for C57 Black mouse, neonatal mouse, days 16 and 20 mouse embryo, with signals strongest in the neonatal heart and day 16 mRNA. The dot blots were positive for spleen and epididymus. It appeared that there was some variability for mouse mRNA expression, unlike human tissue, where heart mRNA consistently was the primarily tissue in which zFGF5 was expressed in humans. Similar variability was seen with rat northern analysis.

Example 14

In vivo Study of Cardiomyopathic Rats

Rats infused subcutaneously with epinephrine for 2 weeks develop a cardiomyopathy quite similar to human idiopathic dilated cardiomyopathy (Deisher et al., Am. J. Cardiovasc. Pathol. 5(1):79-88, 1994 and Deisher et al., J. Pharmacol. Exp. Ther. 266(1):262-269, 1993.)

The effect of zFGF5 on the initiation and progression of the catecholamine-induced cardiomyopathy was evaluated by administering zFGF5 by intra-pericardial injection to male, Sprague-Dawley rats receiving subcutaneous infusions of epinephrine or saline.

In one protocol, rats (300 gms) were implanted with subcutaneous saline- or epinephrine-filled osmotic mini-pumps under light ether anesthesia. 96 hours following minipump implantation, a single intra-pericardial injection of vehicle (n=25) or zFGF5 at 25, 250 or 500 μg/kg was given (n=10 per dose). Mortality was monitored for an additional two weeks, at the end of which the rats were sacrificed, the hearts were weighed wet, and fixed in 10% neutral buffered formalin for histology.

The zFGF5 had no effect on mortality, body weight, heart weight or cardiac fibrosis in saline-infused rats.

In epinephrine-infused rats, the 25 μg/kg and 250 μg/kg doses reduced mortality from 32% in vehicle injected rats to 0% in 25 μg/kg and 10% in 250 μg/kg injected rats. The highest zFGF5 dose, 500 μg/kg, reduced mortality to 20% compared to vehicle injected rats, however this was not statistically significant. Cardiac fibrosis was determined by scoring Masson's Trichrome stained heart sections. Three sections were scored for each heart, and the average score taken. The fibrosis score for the vehicle-infused hearts was 1.26 ± 0.25, while the score for the 25 μg/kg zFGF5 injection was 1.74 ± 0.23, the 250 μg/kg injection was 1.38 ± 0.29, and the 500 μg/kg injection was 0.81 ± 0.10. The dose of zFGF5 which completely prevented mortality increased the cardiac fibrosis score (25 μg/kg), while the dose which had no effect on mortality reduced the cardiac fibrosis score (500 μg/kg). These results indicate that a pro-fibrotic activity can be beneficial in the setting of heart failure of varying etiologies, of which can include myocardial infarct (MI), idiopathic dilated cardiomyopathy (IDCM),

hypertrophic cardiomyopathy, viral myocarditis, congenital abnormalities, and obstructive diseases.

In another protocol, the rats (300 gms) were anesthetized by an intra-muscular injection of an anesthetic cocktail ketamine:rompun:acepromazine (1:1:0.1). Subcutaneous epinephrine-filled osmotic mini-pumps were implanted, and either vehicle or zFGF5 was injected intra-pericardially immediately afterward at 25 μg/kg (n=25 per group). For the vehicle injected rats, 21% had died within 6 days following the epinephrine-filled minipump implantation, while none of the zFGF5 injected rats had died. By the end of the 2 week epinephrine infusion period, 25% of the vehicle-injected rats had died, while only 22% of the zFGF5-injected rats had died. In this model, zFGF5 co-treatment at the time of minipump implantation delayed mortality by at least 7 days.

Example 15

Mammalian Expression Constructs

An expression plasmid containing all or part of a polynucleotide encoding zFGF5 is constructed via homologous recombination. A fragment of zFGF5 cDNA is isolated using PCR that includes the polynucleotide sequence from nucleotide 1 to nucleotide 621 of SEQ ID NO: 1 or SEQ ID NO: 37, with flanking regions at the 5' and 3' ends corresponding to the vectors sequences flanking the zFGF5 insertion point. The primers for PCR each include from 5' to 3' end: 40 bp of flanking sequence from the vector and 17 bp corresponding to the amino and carboxyl termini from the open reading frame of zFGF5.

Ten μl of the 100 μl PCR reaction is run on a 0.8% LMP agarose gel (Seaplaque GTG) with 1 x TBE buffer for analysis. The remaining 90 μl of PCR reaction is precipitated with the addition of 5 μl 1 M NaCl and 250 μl of absolute ethanol. The plasmid pZMP6 which has been cut with SmaI is used for recombination with the PCR fragment.

Plasmid pZMP6 was constructed from pZP9 (deposited at the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, and is designated No. 98668) with the yeast genetic elements taken from pRS316 (deposited at the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, and designated No. 77145), an IRES element from poliovirus, and the extracellular domain of CD8, truncated at the carboxyl terminal end of the transmembrane domain. pZMP6 is a mammalian expression vector containing an expression cassette having the cytomegalovirus immediate early promoter, immunoglobulin signal peptide intron, multiple restriction sites for insertion of coding sequences, a stop codon and a human growth hormone terminator. The plasmid also has an *E. coli* origin of replication, a mammalian selectable marker expression unit having an SV40 promoter, enhancer and origin of replication, a DHFR gene, the SV40 terminator, as well as the URA3 and CEN-ARS sequences required for selection and replication in *S. cerevisiae*.

One hundred microliters of competent yeast cells (*S. cerevisiae*) are independently combined with 10 μl of the various DNA mixtures from above and transferred to a 0.2 cm electroporation cuvette. The yeast/DNA mixtures are electropulsed at 0.75 kV (5 kV/cm), ∞ ohms, 25 μF. To each cuvette is added 600 μl of 1.2 M sorbitol and the yeast is plated in two 300 μl aliquots onto two URA-D plates and incubated at 30°C. After about 48 hours, the Ura+ yeast transformants from a single plate are resuspended in 1 ml H_2O and spun briefly to pellet the yeast cells. The cell pellet is resuspended in 1 ml of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA). Five hundred microliters of the lysis mixture is added to an Eppendorf tube containing 300 μl acid washed glass beads and 200 μl phenol-chloroform, vortexed for 1 minute intervals two or three times,

followed by a 5 minute spin in a Eppendorf centrifuge at maximum speed. Three hundred microliters of the aqueous phase is transferred to a fresh tube, and the DNA precipitated with 600 μl ethanol (EtOH), followed by centrifugation for 10 minutes at 4°C. The DNA pellet is resuspended in 10 μl H_2O.

Transformation of electrocompetent *E. coli* cells (DH10B, GibcoBRL) is done with 0.5-2 ml yeast DNA prep and 40 ul of DH10B cells. The cells are electropulsed at 1.7 kV, 25 μF and 400 ohms. Following electroporation, 1 ml SOC (2% Bacto` Tryptone (Difco, Detroit, MI), 0.5% yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose) is plated in 250 μl aliquots on four LB AMP plates (LB broth (Lennox), 1.8% Bacto Agar (Difco), 100 mg/L Ampicillin).

Individual clones harboring the correct expression construct for zFGF5 are identified by restriction digest to verify the presence of the zFGF5 insert and to confirm that the various DNA sequences have been joined correctly to one another. The insert of positive clones are subjected to sequence analysis. Larger scale plasmid DNA is isolated using the Qiagen Maxi kit (Qiagen) according to manufacturer's instruction.

Example 16

Mammalian Expression of zFGF5

CHO DG44 (Chasin et al., Som. Cell. Molec. Genet. 12:555-666, 1986) are plated in 10 cm tissue culture dishes and allowed to grow to approximately 50 to 70% confluency overnight at 37°C , 5% CO_2, in Ham's F12/FBS media (Ham's F12 medium, (Gibco BRL, Gaithersburg, MD), 5% fetal bovine serum (Hyclone, Logan, UT), 1% L-glutamine (JRH Biosciences, Lenexa, KS), 1% sodium pyruvate (Gibco BRL)). The cells are then transfected with the plasmid zFGF5/pZMP6, using Lipofectamine™ (Gibco BRL), in serum free (SF) media formulation (Ham's F12, 10

mg/ml transferrin, 5 mg/ml insulin, 2 mg/ml fetuin, 1% L-glutamine and 1% sodium pyruvate). ZFGF5/pZMP6 is diluted into 15 ml tubes to a total final volume of 640 μl with SF media. 35 μl of Lipofectamine™ (Gibco BRL) is mixed with 605 μl of SF medium. The Lipofectamine™ mix is added to the DNA mix and allowed to incubate approximately 30 minutes at room temperature. Five milliliters of SF media is added to the DNA:Lipofectamine™ mixture. The cells are rinsed once with 5 ml of SF media, aspirated, and the DNA:Lipofectamine™ mixture is added. The cells are incubated at 37°C for five hours, then 6.4 ml of Ham's F12/10% FBS, 1% PSN media is added to each plate. The plates are incubated at 37°C overnight and the DNA:Lipofectamine™ mixture is replaced with fresh 5% FBS/Ham's media the next day. On day 3 post-transfection, the cells are split into T-175 flasks in growth medium. On day 7 postransfection the cells are stained with FITC-anti-CD8 monoclonal antibody (Pharmingen, San Diego) followed by anti-FITC-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA). The CD8 positive cells are separated by Miltenyi mini-MACS columns according to manufacturer's directions (Miltenyi Biotec), and put into DMEM/Ham's F12/5% FBS without nucleosides but with 50 nM methotrexate (selection medium).

Cells are plated for subcloning at a density of 0.5, 1 and 5 cells per well in 96 well dishes in selection medium and allowed to grow out for approximately two weeks. The wells are checked for evaporation of medium and brought back to 200 μl per well as necessary during this process. When a large percentage of the colonies in the plate are near confluency, 100 μl of medium is collected from each well for analysis by dot blot, and the cells are fed with fresh selection medium. The supernatant is applied to a nitrocellulose filter in a dot blot apparatus, and the filter is treated at 100°C in a vacuum oven to denature the protein. The filter is incubated in 625 mM tris glycine, pH 9.1,, 5mM ßmercaptoethanol, at

65°C, 10 minutes, then in 2.5% non-fat dry milk Western A Buffer (0.25% gelatin, 50 mM TrisHCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% Igepal, Sigma) overnight at 4°C on a rotating shaker. The filter is incubated with the antibody-HRP conjugate in 2.5% non-fat dry milk Western A buffer for 1 hour at room temperature on a rotating shaker. The filter is washed three times at room temperature in PBS plus 0.01% Tween 20, 15 minutes per wash. The filter was developed with ECL reagent according to manufacturer's directions (Amersham, Arlington Heights, IL), and exposed to film (Hyperfilm ECL, Amersham) approximately 5 minutes. Positive clones are trypsinized from the 96 well dish and transferred to 6 well dishes in selection medium for scaleup and analysis by Western blot.

Example 17

Expansion of Cells From Bone Marrow

Assays were performed to measure the frequency of fibroblast colony forming units from monkey low density, non-adherent cells isolated from bone marrow. This assay is indicative of mesenchymal stem cell frequency.

One half of a 96 well microtiter plate is inoculated with cells at a density of 10,000 cells/well and the other half of the plate is inoculated with cells at a density of 1,000 cells/well. The culture medium is α MEM (GIBCO-BRL, Gaithersburg, MD), 2% bovine serum albumin, 10 μg/ml insulin, 200 μg/ml transferrin, antibiotic and 50 μM β-Mercaptoethanol. The cells are incubated at 37°C in 5% CO_2 for 14 days and then stained with toluidine blue to improve cell visibility and examined microscopically. Positive wells have at least 50 cells exhibiting a "stromal" morphology, i.e., large, spread out cells. The positive control is medium containing 20% fetal bovine serum. Results demonstrated that zFGF5, at a concentration of 100 ng/ml increased the

frequency of CFU-F to levels equivalent to the positive control of 20% FBS.

Example 18

Neurite Outgrowth Assay

The effect of zFGF5 on PC12, rat pheochromocytoma cells with neural potential (ATCC No. CRL-1721, American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209) examined using the following growth factors, each at 3 dilutions:

1 µg/ml, 100 ng/ml, and 10 ng/ml neural growth factor (NGF; source and location) in medium containing RPMI 1640 (R&D Systems, Minneapolis, MN)

1 µg/ml, 100 ng/ml, and 10 ng/ml human basic FGF (R&D Systems, Minneapolis, MN)

1 µg/ml, 100 ng/ml, and 10 ng/ml zFGF5 (recombinantly produced in *E. coli.*)

1 µg/ml, 100 ng/ml, and 10 ng/ml zFGF5 (recombinantly produced in CHO cells)

The PC12 cells were plated at a concentration of 5 x 10^4/ml onto collagen coated 24 well culture plates and incubated for 48 hours in the appropriate medium. After 48 hours, the medium was changed to include one of the cytokines described above and then changed again every 2 days. The wells were scored for relative neurite outgrowth on days 6 and 9.

Neurite outgrowth was induced with each of the cytokines. NGF and bFGF appeared to have similar affinity, while zFGF5 had significantly lower affinity. NGF exerted the greatest extent of neural outgrowth activity, followed by bFGF, with significantly lower activity seen with zFGF5.

Example 19

Identification of a Target Cell

Identification of a putative mesenchymal stem cell as a target for zFGF5 was made using FITC-labeled protein and neonatal mouse heart tissue.

ZFGF5, purified as described above, was dialyzed into 0.1 M sodium bicarbonate pH 9.0. Fluorescein isothiocyanate (FITC; Molecular Probes, Eugene, OR) was dissolved at 1 mg/ml in the same buffer without exposure to strong light. The mixture was prepared containing 1 mg FITC/1 mg zFGF5, and reacted for 1-2 hours in the dark at room temperature. The reaction was stopped by adding 1 M glycine to a final concentration of 0.1 M, then reacted for 1 hour at room temperature. The mixture was then dialyzed against 0.1 M sodium biocarbonate to make a 1:500-1:1000 dilution for 3 hours. The dialysis solution was changed and the process repeated for 3-18 hours to remove unlabeled FITC.

Neonatal mouse heart ventricles were isolated, minced, and repeatedly washed in phosphate buffered solution (PBS) until all red blood cells and debris were removed. The minced ventricles were placed in a solution containing 18 ml PBS and 1% glucose and 1 ml of 2% DNAse/Collagenase solution was added. The mixture was incubated on a shaker for 30 minutes at 37°C. The supernatant was discarded and the process was repeated once more. After incubation, the supernatant (~20 ml) was transferred to a tube containing 20 ml DF 20 (Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12, 1:1 (GIBCO-BRL, Gaithersburg, MD) and 20% fetal bovine serum). After mixing, the tubes were centrifuged at 1650 rpms in a Beckman CS-6R centrifuge (Beckman, Fullerton, CA) at 4°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in DF 10 (10% FBS). The cells were kept cold and spun again and resuspended in 40 ml of DF 10. The cell mixture was passed over a 40 µm filter

(Becton Dickinson, Detroit, MI) and counted using a hemacytometer.

The cells were incubated in FITC-labeled zFGF5 at 4°C for 30 minutes at a concentration of 2 x 10^6 cells/1 μg zFGF5. After incubation, the cells were spun at 1650 rpms in a Beckman CS-6R centrifuge (Beckman) for 5 minutes. The supernatant was discarded and the pellet washed once in 10 ml of DF 10 and resuspended in 4 ml DF 10.

10 μl of MACS anti-FITC microbeads (Miltenyi Biotech, Auburn, CA) were mixed with 10^7 cells in 4 ml of DF10 and incubated at 4°C for 30 minutes.

MACS positive selection type LS+ separation columns (Miltenyi Biotech) were washed with 3 ml of MAC buffer (PBS, 0.5% BSA, 2 mM EDTA) and the cell/bead mixture was washed in 10 ml MAC buffer and then resuspended in 6 ml MAC buffer. The cell/bead mixture was divided between the two columns and the first negative fraction was discarded. 1.5 ml of 0.6 M NaCl was added to each column and eluted but not collected. The columns were then washed with 1.5 ml MAC buffer. The cells bound with FITC-labeled zFGF5 were collected by adding 3 ml MAC buffer, removing the column from the magnet and flushing out the positive cells using the plunger. The positive cell fraction was plated in a T75 flask and 50 ml of plating medium was added (DF with 15% FBS and antibiotics). The cells were incubated at 37°C for 1 week and counted. The yield of positive cells was approximately 0.1% of original total cells counted.

Cells binding FITC-labeled zFGF5 were examined by transmission electronmicroscopy (TEM). The cells were between 3-5 microns in diameter. The cell nuclei occupied the majority of the cell volume, and few cytoplasmic organelles were apparent. The phenotype identified by TEM identifies the zFGF5-isolated cells as primitive mesenchymal stem cells.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.